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U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES
DESIGNATED/ELECTED OFFICE (DO/EO/US)
CONCERNING A FILING UNDER 35 U.S.C. 371ATTORNEY'S DOCKET NUMBER
SHIM012U.S. APPLICATION NO.
To Be Assigned

09/936883

INTERNATIONAL APPLICATION NO.
PCT/JP00/01646INTERNATIONAL FILING DATE
17 March 2000PRIORITY DATE CLAIMED
19 March 1999 and 28 October 1999

TITLE OF INVENTION

A METHOD FOR DETECTING MEGSIN PROTEIN AND USE THEREOF

APPLICANT(S) FOR DO/EO/US

Toshio Miyata

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This express request to begin examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and the PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2)):
 - a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☒ has been transmitted by the International Bureau. (**However, a courtesy copy is attached hereto.**)
 - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (37 U.S.C. 371(c)(3)):
 - a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☐ have been transmitted by the International Bureau.
 - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
 - d. ☒ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)). (**UNSIGNED**)
10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11 to 16 below concern other document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☐ A **FIRST** preliminary amendment.
☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ☒ Other items or information: Certification Regarding Sequence Listing ; Paper Copy of Sequence Listing ; Diskette containing sequence listing; Return Receipt Postcard

U.S. APPLICATION NO. (If known, see 37 CFR 1.50) To Be Assigned 09/936883		INTERNATIONAL APPLICATION NO. PCT/JP00/01646		ATTORNEY'S DOCKET NUMBER SHIM012	
17. <input checked="" type="checkbox"/> The following fees are submitted: Basic National Fee (37 CFR 1.492(a)(1)-(5)): Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and international search report not prepared by the EPO or JPO \$1,0000.00 International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search report prepared by the EPO or JPO \$860.00 International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO \$690.00 International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) \$710.00 International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) \$ 100.00					
ENTER APPROPRIATE BASIC FEE AMOUNT =				\$ 860.00	
Surcharge of \$130.00 for furnishing the oath or declaration later than months from the earliest claimed priority date (37 CFR 1.492(e)).				\$ 0.00	
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total Claims	18 - 20 =	0	X \$18.00	\$ 0.00	
Independent Claims	5 - 3 =	2	X \$80.00	\$ 160.00	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ \$260.00	\$ 0.00	
TOTAL OF ABOVE CALCULATIONS =				\$ 1020.00	
Applicant claims small entity status. See 37 CFR 1.27. Reduction of 1/2 for filing by small entity.				\$ 0.00	
SUBTOTAL =				\$ 1020.00	
Processing Fee of \$130.00 for furnishing the English translation later than months from the earliest claimed priority date (37 CFR 1.492(f)).				\$ 0.00	
TOTAL NATIONAL FEE =				\$ 1020.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property.				\$ 0.00	
TOTAL FEES ENCLOSED =				\$ 1020.00	
				Amount to be: refunded	\$
				charged	\$1020.00

- a. A check in the amount of \$ * to cover the above fees is included.
- b. ... ☒ Please charge my Deposit Account No. 50-0815 in the amount of \$ 1020.00 to cover the above fees. A duplicate copy of this sheet is enclosed.
- c. ... ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 50-0815. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO

BOZICEVIC, FIELD & FRANCIS LLP
200 Middlefield Road, Suite 200
Menlo Park, CA 94025
(650) 327-3400 Telephone
(650) 327-3231 Facsimile
.....

SIGNATURE

Karl Bozicevic

NAME

28.807

REGISTRATION NO.

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**CERTIFICATION
REGARDING SEQUENCE
LISTING &
PRELIMINARY
AMENDMENT**

Address to:
BOX SEQUENCE
Assistant Commissioner for Patents
PO BOX 2327
Arlington, VA 22202

Attorney Docket	SHIM-012
First Named Inventor	TOSHIO MIYATA
Application Number	09/936,883
Intl. Filing Date	17 March 2000
Confirmation Number	2901
Group Art Unit	To Be Assigned
Examiner Name	To Be Assigned
Title: "A METHOD FOR DETECTING MEGSIN PROTEIN AND USE THEREOF"	

Sir:

This Certification is in response to the Notification of Defective Response mailed on March 27, 2002 for which a one month period for response was given making this response timely filed on or before April 27, 2002.

This is a combined Sequence Listing Certification and Preliminary Amendment to the patent application identified above. Prior to examination of the subject application, please enter the following amendment(s):

AMENDMENTS

In the Specification:

Please insert the following paragraph(s) just prior to the section with the heading, "Background of the Invention":

SEQUENCE LISTING

The present specification incorporates herein by reference, each in its entirety, the sequence information on the Compact Disks (CDs) labeled Copy 1 and Copy 2. The CDs are formatted on IBM-PC, with operating system compatibility with MS-Windows. The files on each of the CDs are as follows:

Copy 1 - Seqlist.txt 11 KB created April 5, 2002; and

Copy 2 - Seqlist.txt 11 KB created April 5, 2002.

09936883-123101
F072T E88E60

REMARKS

Formal Matters

The present application has been amended under 37 C.F.R. §§ 1.52(e)(5) and 1.77(b)(4) to incorporate-by-reference sequence listing information submitted herewith in electronic format.

Attached hereto is a marked-up version of the changes made to the present application by the current amendment. The attached is captioned "**Version with Markings to Show Changes Made**".

No new matter is added.

Certification Regarding Sequence Listing

I hereby certify that the enclosed submission contains no new matter. As required by 37 § C.F.R. 1.821(f), all information on CD 'Copy 1', 'Copy 2' and 'CRF' is identical. I hereby certify that the enclosed submission includes no new matter. The Sequence Listing was prepared with the software FASTSEQ, and conforms to the Patent Office guidelines. Applicant respectfully submits that the subject application is in adherence to 37 CFR §§ 1.821-1.825.

The Commissioner is hereby authorized to charge any underpayment of fees associated with this communication, including any necessary fees for extensions of time, or credit any overpayment to Deposit Account No. 50-0815, order number SHIM-012.

Respectfully submitted,

Dated: 5/April/2002

By: Karl Bozicevic

Registration No. 28,807

BOZICEVIC, FIELD & FRANCIS LLP
200 Middlefield Road, Suite 200
Menlo Park, CA 94025
Telephone: (650) 327-3400
Facsimile: (650) 327-3231

“Version with Markings to Show Changes Made”

The following paragraph has been added after the section titled, ‘Background of the Invention’:

SEQUENCE LISTING

The present specification incorporates herein by reference, each in its entirety, the sequence information on the Compact Disks (CDs) labeled Copy 1 and Copy 2. The CDs are formatted on IBM-PC, with operating system compatibility with MS-Windows. The files on each of the CDs are as follows:

Copy 1 - Seqlist.txt 11 KB created April 5, 2002; and

Copy 2 - Seqlist.txt 11 KB created April 5, 2002.

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EL923479292US

DESCRIPTION

A METHOD FOR DETECTING MEGSIN PROTEIN AND USE THEREOF

5 Technical Field

The present invention relates to a method for evaluating renal function, which comprises measuring the megsin protein in biological specimens such as urine, blood, and so on. Furthermore, the present invention relates to reagents for diagnosing renal function comprising anti-megsin protein antibodies.

Background Art

Kidney transplantation is the only method to treat terminal renal failure where the renal functions of hemofiltration and detoxication fail completely. However, the supply system supporting renal transplantations in Japan is far from well structured. In addition, social cognition on the transplantation method itself is not advanced. Therefore, the present status is that there is no alternative but to rely on dialysis as a renal replacement treatment.

Today, the number of patients who undergo dialysis is estimated to be approximately 170,000. An average annual treatment cost of about ¥6 million is needed per patient, and it is considered to be one of the biggest reasons that constrain the health insurance system. Furthermore, treatment with dialysis restraints the patient for 4 to 6 hours a day, 2 to 3 days a week, which greatly hampers the social activities of the patient.

Renal failure is a condition patients with renal diseases finally reach. The cause and background leading to renal failure is diverse. There are many cases in which diseases that usually originate outside the kidneys such as drug poisoning, infectious diseases, malignant tumors, diabetes, systemic lupus erythematosus (SLE), cause renal disorders and finally lead to renal failure.

Significant subjective symptoms of renal disorders appear only at late stages, namely only after the condition has almost worsened to renal failure. Therefore, they are easily overlooked, and

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numerous cases exist where at the point when symptoms appear, the kidney has already suffered an unrecoverable damage. Thus, it is important to discover renal disorders at an earlier stage as possible before the expression of subjective symptoms in order to prevent transition to renal failure, and also to eliminate constraints dialysis treatment puts on public medical insurance finances.

Up to now, the examination of urinary proteins and urinary deposits, the so-called urinalysis, was widely carried out to diagnose renal disorders. However, urinary proteins increase temporarily even in normal healthy persons due to extreme exercise, psychological stress, abundant meat diet, before menstruation, and so on. On the other hand, there are urinary proteins that are unrelated to renal diseases, such as orthostatic albuminuria found mainly in adolescents (in around 0.5% of normal healthy persons). Urinary proteins are also detected in uropathies, bladder disorders, female genital tract disorders, and so on. Thus, it is difficult to make a definite diagnosis of renal disorders only by examining urinal proteins.

Urine deposits are observed with a microscope after centrifugation of urine. However, erythrocyte deposits are also observed in the urine deposits of normal healthy persons, and may also derive from disorders other than renal disorders, such as those of organs related to the urinary tract. Therefore, urine deposits are also not sufficient enough to make definite diagnoses of renal disorders.

A method to diagnose diabetic nephropathy at an early stage is known, which comprises the following steps: (1) assaying albumin that leaked into the urine as an indicator of diabetic nephropathy; and (2) comparing the value with the normal value of a normal healthy person. However, the exact state of diabetic nephropathy cannot be understood since the albumin content in the urine changes even in a normal healthy person.

In addition, serum creatinine (Cr) and blood urea nitrogen (BUN) are assayed to examine the retention of urinary components in the blood, but this assay is also easily influenced by diet. Thus, abnormal values in assays of urinary proteins, as well as serum Cr and BUN does not necessarily imply a renal disorder, and they may

frequently occur in normal healthy persons or patients with other diseases.

Further, diagnosis of renal disorders by measuring various substances including urinary β_2 -microglobulin, N-acetylglucosaminidase (NAG), IgG, transferrin, interleukin-6, and such are being tried. However, there are many cases where these measurements do not correspond to the severity of the renal disorder, and therefore, are far from being effective.

Moreover, although a disorder of the whole kidney or involvement of immune reactions may be guessed by measuring these blood components in the urine, it is difficult to identify the site within renal tissue that is affected by the disorder. No examination method other than those mentioned above is known for diagnosing renal disorders and determining the severity with a sufficient sensitivity and specificity. Histological diagnosis by renal biopsy is regarded essential for ultimately diagnosing and determining the severity of renal disorders.

However, renal biopsy is an invasive examination, and frequently accompanies dangers of complications such as hemorrhage, infections, and so on. Further, to conduct the examination, hospitalization in a well-equipped facility having specialists is required, and the physical and social burden on the patient cannot be ignored.

As mentioned above, the examination by urinalysis is simple and convenient, and is also an excellent examination method that enables treatment of a large amount of specimens. However, from the perspective of providing a definite diagnosis of renal disorders, it is far from being satisfactory. On the other hand, renal biopsy is an authentic method to diagnose and determine the severity of renal disorders. However, its use is highly restricted, which cannot be helped. On this account, a method to diagnose renal disorder, which has the simplicity and convenience of a urinalysis, as well as the accuracy of a renal biopsy, has been desired.

Proteins specifically expressed in specific tissues, not only those in the kidney, are often used as indicators of functional disorders of the organ. For example, enzymatic proteins such as LDH

and γ GTP are widely used as markers of hepatic function. However, no protein specific to the kidneys is known which serves as an indicator of their functions.

The present inventors isolated a gene called megsin as a gene especially strongly expressed in mesangial cells by a macroscale DNA sequence analysis and database analysis, and succeeded in obtaining the megsin protein comprising 380 amino acids encoded by the megsin full-length cDNA clone. The present inventors, further found that the human megsin protein belongs to the SERPIN (serine protease inhibitor) superfamily (R.Carrell et al., Trends Biochem.Sci., 10:20 (1985); R.Carrell et al., Cold Spring Harbor Symp.Quant.Biol., 52:527 (1987); E.K.O.Kruithof et al., Blood, 86:4007 (1995); J.Potempa et al., J.Biol.Chem., 269:15957 (1994); E.Remold-O'Donnell, FEBS Let., 315:105 (1993)) according to an amino acid homology search by the FASTA program using the Swiss Prot database (T.Miyata et al. J.Clin.Invest., 120:828-836 (1998)). These findings were filed as a patent application (PCT/JP98/04269).

Human megsin protein expression was weak in human fibroblasts, smooth muscle cells, endothelial cells, and keratinocytes, and was strong especially in mesangial cells. The comparison of megsin protein expression level in renal tissues from IgA nephropathy patients and normal healthy individuals revealed that the expression level of megsin protein in IgA nephropathy patients was significantly higher. Accordingly, it is possible that megsin protein existing in the urine or blood is useful as a marker of mesangial cell proliferative nephropathy, for example, nephropathies such as IgA nephropathy. There is a possibility that megsin protein gene expression is deeply related with the onset and progression of renal diseases. However, the correlation between megsin protein gene expression and progression of renal disease state was unknown.

Disclosure of the Invention

An objective of the present invention is to solve problems such as those above, and to provide a method that enables diagnosis of renal diseases and determination of their severity, and also to provide reagents and reagent kits used for the method.

Firstly, the present inventors thought that it is necessary to measure specific proteins related to the state of the disease to make a definite diagnosis of renal disorders and determine their severity, and focused their attention on mesangial cells, which exist in the glomerulus. The mesangium is mapped on the lobule center in the glomerular tuft and is a tissue that functions as a core to bind the lobules together. The mesangium is covered by the glomerular basement membrane. It is composed of cells (mesangial cells), which are divided from the capillary lumen by endothelial cells, and a three-layered amorphous material (mesangial matrix), which is in sequence with the lamina rara interna in the glomerular basement membrane.

The mesangial cells play a pivotal role in maintaining the structure and function of the glomerulus. Their proliferation is thought to be a main factor in the onset of glomerular disorders such as glomerular nephritis and glomerulosclerosis. Furthermore, mesangial cells are targets damaged by all types of nephritis. For example, proliferation of mesangial cells and accumulation of extracellular mesangial matrix is thought to be the first step in the development of glomerulosclerosis in patients suffering from various glomerular diseases, such as chronic nephritis and diabetic nephritis, the two main causes of end-stage renal failure(D. Schlondorff, Kidney Int., 49: 1583-1585, (1996); R. B. Sterzel et al., Glomerular mesangial cell. Immunologic Renal Diseases, 595-626 (1997)) Therefore, it is thought that identification of a gene expressed specifically in mesangial cells and elucidation of its function would be helpful in understanding the biological characteristics of mesangial cells and the causes of diseases relating to mesangial cells, and in turn, treating or diagnosing diseases related to mesangial cells.

It has been reported that steroidal agents are effective for IgA nephropathy with an extreme proliferation of mesangial cells (J.V.Donadio et al., J.Am.Soc.Nephrol. 8:1324-1332 (1997)). However, use of steroidal agents often causes serious side effects, and thus, aimless administration of steroidal agents to types of IgA nephropathy for which steroidal agents are ineffective, should be avoided.

Therefore, a matching up with the severity of the tissue such as the degree of proliferation of mesangial cells, and so on, is essential for steroid therapy. Accordingly, a renal function assay that can simply and conveniently evaluate the degree of proliferation of mesangial cells was long desired.

The present inventors assumed that megsin protein gene expression increases in relation to the onset and progress of renal diseases, and in association thereto, megsin protein production increases and leads to a leakage of megsin protein into urine or blood, the amount of which may be related to the progression of disease state. To verify the mechanism, the inventors attempted to measure and compare the concentration or amount of megsin protein in various biological specimens, and thus, accomplished the present invention through the result that renal diseases related to megsin protein can be evaluated by measuring the concentration or amount of the megsin protein. Thus, the present invention relates to a method for evaluating renal functions, as well as to reagents or reagent kits for the method, which are as follows:

- (1) a method for evaluating renal functions, which comprises measuring the megsin protein in a biological specimen;
- (2) the method for evaluating renal functions of (1), which comprises measuring the megsin protein in a biological specimen, and comparing it with the megsin protein amount in a normal specimen;
- (3) the method for evaluating renal functions of (1), wherein the biological specimen is urine;
- (4) the method for evaluating renal functions of (1), wherein the megsin protein in the biological specimen is measured by an antigen-antibody reaction using an anti-megsin protein antibody;
- (5) the method for evaluating renal functions of (4), wherein the anti-megsin protein antibody is a monoclonal antibody;
- (6) a reagent for diagnosing renal functions, which comprises the anti-megsin protein antibody;
- (7) the reagent for diagnosing renal functions of (6), wherein the anti-megsin protein antibody is a monoclonal antibody;
- (8) a granule for detecting megsin protein in a biological specimen, wherein the granule comprises a solid granule to the surface of which

an anti-megsin protein antibody is bound;

(9) the granule for detecting megsin protein of (8), wherein the solid granule is magnetic;

(10) the granule for detecting megsin protein of (8), wherein the relative density of the solid granule is not smaller than 1;

(11) the granule for detecting megsin protein of (8), wherein the anti-megsin protein antibody is a monoclonal antibody;

(12) a method for detecting megsin protein in a biological specimen, comprising the following steps of:

i) contacting the granule for detecting megsin protein with the biological specimen, said granule comprising a solid granule to the surface of which an anti-megsin protein antibody is bound;

ii) contacting the second anti-megsin protein antibody bound to a marker molecule with said granule for detecting megsin protein to which the biological specimen was contacted; and,

iii) detecting the marker molecule bound to the megsin protein through the second anti-megsin protein antibody;

(13) the method for detection of (12), wherein the first anti-megsin protein antibody and the second anti-megsin protein antibody are both monoclonal antibodies;

(14) the method for detection of (13), wherein the first anti-megsin protein antibody and the second anti-megsin protein antibody are antibodies having different recognition sites;

(15) the method for detection of (12), wherein the biological specimen is urine;

(16) the method for detection of (12), wherein the biological specimen is blood;

(17) a kit for detecting megsin proteins, which comprises the following elements:

a) magnetic solid granules to which anti-megsin protein antibodies can be bound,

b) anti-megsin protein antibodies, which are bound to said magnetic solid granules in advance, or can be bound to them indirectly, and

c) a magnet;

(18) the kit for detecting megsin proteins of (17), further comprising an anti-megsin protein antibody to which a marker molecule is bound.

As mentioned above, the megsin protein was isolated as a protein encoded by a gene that is highly expressed in human kidney mesangial cells. The megsin protein of the present invention includes not only a protein having the amino acid sequence of SEQ ID NO: 2 (human megsin protein), but also proteins functionally equivalent thereto. For example, megsin protein homologues from species other than human can be mentioned as proteins that are functionally equivalent. Other examples are precursors and fragments of these homologues. Megsin protein-protease complexes can also be used as indicators of renal functions, as long as they can be detected within biological specimens. Megsin protein homologues of species other than humans can be used as indicators for the evaluation of renal functions in the respective species. Thus, they are useful to understand disease states in renal function disorder models.

Megsin protein homologues from species other than human can be obtained, for example, as follows: First, the homologue cDNA is amplified by PCR utilizing a degenerative primer designed based on the nucleotide sequence of the cDNA encoding the human megsin protein of SEQ ID NO: 1 to correspond to the beginning and end of the ORF. In case where the amplified cDNA is not a full-length cDNA, its upstream nucleotide sequence can be determined by methods such as 5'RACE. For example, RT-PCR is conducted using the mRNA, which is extracted from cultured mesangial cells, as the template for rats. Degenerative primers used can be, for example, the oligonucleotides with the following nucleotide sequences. Part of the structure of the rat homologue megsin protein can be revealed by sequence determination of the clone obtained by such a PCR method. Degenerative primer FY: GTGAATGCTGTGTACTTAAAGGCAANTGN/ SEQ ID NO: 3 (corresponding to 172VNAVYFKGK180) Degenerative primer R21: AANAGRAANGGRTCNGC/ SEQ ID NO:4 (wherein R is A or G; corresponding to 357ADHPFLF363)

However, to obtain the 5' regions, it is necessary to conduct a degenerate PCR again by preparing gene specific primers from clone fragments of the rat megsin protein, since only a part of the cDNA can be amplified with these primer pairs. The nucleotide sequences of the primers used, which correspond to the N terminal amino acid

sequences of the megsin proteins are as follows:

Degenerative primer RM-CtermC1: ATGGCNTCNGCNGCNGCNGCNAAYGC/ SEQ ID NO:5 (wherein Y is T or C; corresponding to the sequence encoding the N-terminus of the megsin protein)

5 RM-MR-A2: CGACCTCCAGAGGCAATTCCAGAGAGATCAGCCCTGG/ SEQ ID NO: 6

RM-MR-A1: GTCTTCCAAGCCTACAGATTTCAAGTGGCTCCTC/ SEQ ID NO: 7

(Each of them is a rat megsin protein specific reverse primer)

The 3' region of the cDNA encoding the rat homologue can be derived by inserting the obtained PCR product into the pGEM-T-easy
10 vector (Promega) to determine the nucleotide sequence. Finally, the nucleotide sequence of the full-length cDNA encoding the homologue can be determined by conducting 5'-RACE and 3'-RACE using a primer designed based on the nucleotide sequence obtained as above. Two gene specific antisense primers, namely RM-PRO1:
15 GCTCAGGGCAGTGAAGATGCTCAGGGAAGA/ SEQ ID NO:8 and RM-PRO2: CTGACGTGCACAGTCACCTCGAGCACC/ SEQ ID NO:9, can be used for the 5'-RACE for rats. On the other hand, gene specific sense primer RM-MR-S3: GAGGTCTCAGAAGAAGGCACTGAGGCAACTGCTGCC/ SEQ ID NO:10 can be used in the 3'-RACE. Mouse homologues can be isolated by the same method.
20 The sequence of rat and mouse megsin cDNA nucleotide sequence depicted in SEQ ID NO:18 (rat) and SEQ ID NO:20 (mouse) were determined according to the method above.

The method used in the present invention to measure the megsin protein is not restricted. For example, immunological assays, which
25 make use of the immunological reaction between an antibody for the megsin protein and the megsin protein, are excellent in both specificity and sensibility. Such examples are immunoprecipitation, radioimmunoassay, immunofluorescence assay, enzyme immunoassay, chemiluminescence assay, and immunohistochemistry. Furthermore,
30 the megsin protein can be measured by the Western Blot method utilizing an antibody against the megsin protein. These immunoassays can also be combined, such as performing the Western Blot method after the immunoprecipitation. These assay methods are well known in the technical field of the invention.

35 Immunohistochemistry is a method where the antibody of the present invention is reacted with an isolated human cell or disrupted

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cell solution thereof, tissue or disrupted tissue solution thereof, blood serum, pleural effusion, ascites, tears, and such, and further reacted with the anti-mouse IgG antibody or binding fragments, which are labeled with fluorescent substances like fluorescein isothiocyanate (FITC) or with enzymes like peroxidase, and then are observed using a microscope. Each marker substance can be also labeled indirectly by binding streptavidin bound marker substances with biotinylated antibodies. Furthermore, since the megsin protein is a protease inhibitor, it can be detected using its inhibition activity on protease as the indicator. Alternatively, the megsin protein can be assayed utilizing its affinity towards the protease.

The source or preparation method of the antibody, which is essential for immunological assays of the megsin protein, can be any so long as the antibody can recognize the detection target, which is the megsin protein. Thus, antibodies to be used include polyclonal antibodies, monoclonal antibodies, mixtures thereof, etc. Furthermore, fragments containing the variable region of the antibody molecule are included. The antibody against megsin protein can be obtained, for example, as follows. For example, antibodies used in the present invention comprise those antibodies raised against the protein having the amino acid sequence of SEQ ID NO:2. The antibody (for example, polyclonal antibody or monoclonal antibody) or the antiserum against the megsin protein or partial amino acids sequence thereof can be prepared according to conventional methods for antibody or antiserum preparation using megsin proteins, oligopeptides comprising a partial amino acid sequence thereof, as well as fusion proteins, such as c-myc-(His)₆-Tag-megsin protein and MBP-megsin protein, as the antigens. For example, monoclonal antibodies can be prepared following the methods below. In cases where synthetic peptides with partial amino acid sequence are used as immunogens, it is generally convenient to use highly hydrophilic amino acid sequences that are specific to megsin protein. Such amino acid sequences include those shown in SEQ ID NO:11 to 17, which were used in the examples. Among these sequences, the amino acid sequences shown in SEQ ID NO:12 (human), SEQ ID NO:14 (human), or SEQ ID NO:17 (rat) especially yielded antibodies with good reactivity to the

glomerulus tissue.

The megsin protein of the present invention or a synthetic peptide having a partial amino acid sequence of the present megsin protein, is administered alone or together with a carrier or diluent to a warm-blooded animal at a site capable of producing antibodies. Synthetic peptides used as immunogens are in the form peptides bound to carrier proteins such as bovine thyroglobulin and keyhole limpet hemocyanin. To enhance the antibody productivity, the complete Freund's adjuvant or incomplete Freund's adjuvant can be administered together with the antigen. Immunization is performed once every 1 to 6 weeks, a total of about 2 to 10 times, in general. Warm-blooded animals to be used are, for example, monkeys, rabbits, dogs, guinea pigs, mice, rats, sheep, goats, and domestic fowls, preferably mice or rats. Monoclonal antibody-producing cells can be prepared by selecting immunized warm-blooded animals, such as mice, in which an antibody titer is detected, obtaining the spleen or lymph nodes from the animals 2 to 5 days after the final immunization, and fusing the antibody producing cells contained in these tissues with myeloma cells. Reacting the labeled megsin protein described below with the antiserum, and measuring the activity of the label bound to the antibody enables measurement of the antibody titer in the antiserum.

A monoclonal antibody of the present invention that does not crossreact with proteins other than megsin protein can be obtained by selecting an antibody that recognizes epitopes specific to megsin protein. In general, an epitope specific to the protein comprises at least 7 or more continuous amino acid residues, preferably 10 to 20 amino acids in the amino acid sequence of the protein. Therefore, for example, a monoclonal antibody recognizing an epitope comprising peptides having an amino acid sequence selected from the amino acid sequence of SEQ ID NO: 2, SEQ ID NO: 19 (rat), or SEQ ID NO: 21 (mouse), and comprising at least 7 continuous amino acid residues can be a monoclonal antibody specific to the megsin protein. Conserved amino acid sequences among the amino acid sequences of SEQ ID NO: 2, SEQ ID NO: 19 (rat), and SEQ ID NO: 21 (mouse) can be selected to choose epitopes common to the megsin protein family. If a region contains amino acid sequences specific to all sequences, a monoclonal antibody

capable of recognizing different species can be selected.

An anti-MEGSIN protein monoclonal antibody can be separated and purified according to a separation and purification method for immunoglobulin, similar to the separation and purification of polyclonal antibodies. The known purification methods include, for example, salting out, alcohol precipitation, isoelectric point precipitation, electrophoresis, adsorption and desorption method by ion exchangers (for example, DEAE), ultra centrifugation, gel filtration, or specific purification methods whereby an antibody is exclusively collected, for example, methods whereby an antigen is collected using an antigen binding solid phase or active adsorbent, such as Protein A or Protein G, and thereafter the binding is dissociated to obtain the antibody.

Monoclonal antibodies and polyclonal antibodies obtained in such a manner that recognize the megsin protein of the present invention, can be used for the diagnosis and treatment of diseases relating to mesangial cells. Examples of methods that use these antibodies to measure the megsin protein include a sandwich assay, which comprises reacting the megsin protein with an antibody binding to an insoluble carrier and a labeled antibody and detecting MEGSIN protein in the sandwiched complex produced by the reaction, or a competitive method comprising competitively reacting labeled human urine-derived megsin protein and human urine-derived megsin protein in a sample with an antibody to measure human urine-derived megsin protein in the sample based on the labeled antigen amount that reacted with the antibody.

The assaying of human urine-derived megsin protein by the sandwich method is conducted by a 2 step method in which an immobilized antibody is reacted with human urine-derived megsin protein, unreacted materials are completely removed by washing, and a labeled antibody is added to form the immobilized antibody-labeled human urine-derived MEGSIN protein antibody complex, or one step method in which the immobilized antibody, the labeled antibody, and human urine-derived MEGSIN are mixed at the same time.

Examples of insoluble carriers used for the assay include, for example, polystyrene, polyethylene, polypropylene, polyvinyl chloride, polyester, polyacrylate, nylon, polyacetal, synthetic

resin such as fluoride resin, etc., polysaccharides such as cellulose, agarose, etc., glass, metals, etc. The form of the insoluble carrier can be varied and includes a shape like a particle, tray, sphere, fiber, stick, board, container, cell, test tube, etc. The antibody-adsorbed carrier should be stored in a cool place in the presence of appropriate preservatives, such as sodium azide.

On the other hand, solid granules are known as reagents used to evaluate the fertility of human sperm cells. These granules comprise on their surfaces monoclonal antibodies that specifically react with human sperm cells having undergone the acrosome reaction (Japanese Patent No.2651249). This granule used for sperm fertility detection is a solid granule to which a monoclonal antibody that specifically reacts with human sperm cells having undergone the acrosome reaction is bound. The fertility of human sperm cells can be evaluated by reacting sperm cells with the granules and counting sperm cells bound to the granules. The fertility can be tested readily and safely without the use of complicated techniques like radioactivity, fluorescence, and so on. Samples of small quantities can be assayed by using magnetic solid granules since the granules can be aggregated easily by using a magnet. The present inventors found out that it is possible to detect megalin protein more easily and accurately by applying this technique.

Antibodies can be immobilized by known chemical binding or physical adsorption methods. Chemical binding methods include, for example, a method using glutaraldehyde, the maleimide method using N-succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate, N-succinimidyl-2-maleimidoacetate, etc., and the carbodiimide method using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride, etc. Further examples are, the maleimidobenzoyl-N-hydroxysuccinimidoester method, the N-succinimidyl-3-(2-pyridyldithio)propionate method, the bisdiazolated benzidine method, and dipalmitoyllysine method. Alternatively, a complex, which is prepared by reacting two different antibodies having different epitopes with the substance to be detected, can be captured with a third antibody immobilized by the above method.

The label is not limited, so long as it is useful in immunoassays. Specifically, enzymes, fluorescent substances, luminescent substances, radioactive substances, metal chelates, etc. can be used. Preferable labeling enzymes are, for example, peroxidase, alkaline phosphatase, β -D-galactosidase, malate dehydrogenase, *Staphylococcus* nuclease, delta-5-steroid isomerase, α -glycerol phosphate dehydrogenase, triosephosphate isomerase, horseradish peroxidase, asparaginase, glucose oxidase, ribonuclease, urease, catalase, glucose-6-phosphate dehydrogenase, glucoamylase, and acetylcholine esterase, etc. Preferable fluorescent substances include, for example, fluorescein isothiocyanate, phycobiliprotein, rhodamine, phycoerythrin, phycocyanin, allophycocyanin, and orthophthalaldehyde. Preferable luminescent substances include, for example, isoluminol, lucigenin, luminol, aromatic acridinium ester, imidazole, acridinium salt and its modified ester, luciferin, luciferase, and aequorine. Preferable radioactive substances include, for example, ^{125}I , ^{127}I , ^{131}I , ^{14}C , ^3H , ^{32}P , ^{35}S , etc.

Methods for binding the above labels are known. Specifically, direct and indirect labeling can be used. The commonly used direct labeling method is the method in which an antibody or an antibody fragment is chemically covalent-bound to a label using a cross-linking agent. Cross-linking agents include N,N'-orthophenylenedimaleimide, 4-(N-maleimidomethyl) cyclohexanoate N-succinimide ester, 6-maleimidohexanoate N-succinimide ester, 4,4'-dithiopyridine, and other known crosslinking agents. The crosslinking agent can be reacted with enzymes and antibodies by known methods depending on the characteristics of the cross-linking agent. An example of the indirect labeling method comprises binding an antibody to a low molecular weight hapten such as biotin, dinitrophenyl, pyridoxal, or fluorescamine, and indirectly labeling the antibody with the binding partner to the hapten. Avidin and streptavidin can be used as a recognition ligand for biotin, whereas dinitrophenyl, pyridoxal, or fluorescamine are labeled with antibodies recognizing these haptens.

Horseradish peroxidase can be used as an enzyme for labeling antibodies. This enzyme is useful because it can react with many

substrates and be easily bound to antibodies by the periodate method. Occasionally, antibody fragments, for example, Fab', Fab, F(ab')₂ are used as antibodies. Both polyclonal and monoclonal antibodies can be labeled with an enzyme by the same method. Enzyme-labeled antibodies obtained using the above crosslinking agent can be purified by known methods such as affinity chromatography, etc. to serve as more sensitive immunoassay systems. Purified enzyme-labeled antibodies are stored with a preservative such as thimerosal and a stabilizer such as glycerol. Labeled antibodies can be lyophilized and stored in the cool and dark place for a long time.

When the label is an enzyme, its substrate and, if necessary, a coloring agent is used for measuring its activity. When peroxidase is used as an enzyme, H₂O₂ is used as the substrate solution, and 2,2'-azino-di-[3-ethylbenzothiazolinesulfonic acid] ammonium salt (ABTS), 5-aminosalicylic acid, orthophenylenediamine, 4-aminoantipyrine, or 3,3', 5,5'-tetramethylbenzidine, etc. are used as coloring agents. When alkaline phosphatase is used as the enzyme, orthonitrophenylphosphate, paranitrophenylphosphate, etc. can be used as substrates. When β-D-galactosidase is used as the enzyme, fluorescein-di-(β-D-galactopyranoside), 4-methylumbelliferyl-β-D-galactopyranoside, etc. can be used as substrates. The present invention also includes an immunoassay reagent for megsin comprising labeled or immobilized monoclonal or polyclonal antibodies. The present invention further includes a kit comprising this reagent and an indicator for detection labels and a control sample, etc.

Any biological sample such as body fluid including blood plasma, serum, blood, urine, tissue fluid, or cerebrospinal fluid, etc. can be used as samples for measuring the megsin protein of the present invention, as long as they contain megsin protein or its precursor or a fragment. Among these biological samples, megsin protein can be detected with high frequency especially in urine, accompanied by a proliferation and activation of mesangial cells. Therefore, measurement of megsin in urine is useful as a marker for mesangial proliferative nephropathy, such as IgA nephropathy.

Evaluation of renal functions as used herein means to comprehend

the condition of mesangial cells, which is an important cell constructing kidney tissue, and to determine the presence or severity of a renal disease causing abnormalities in mesangial cells. Renal disease as used herein refers to any disease causing an abnormality in mesangial cells. More specifically, IgA nephropathy, acute glomerulonephritis, focal sclerosing glomerulopathy, membranoproliferative glomerulonephritis, diabetic nephritis, lupus nephritis, and so on, can be mentioned as such diseases. The condition of renal functions affected by these renal diseases can be evaluated by the method of the present invention. Among the above diseases, the present method for evaluating renal function is especially useful as a marker for mesangial cell proliferative nephropathy, such as the IgA nephropathy. Moreover, the method of the present invention for evaluating renal function may be applied not only to determine the existence or severity of a renal disease, but also to evaluate a therapeutic effect or make a prognosis. In addition, the method of the present invention for evaluating renal function enables the exclusion of diseases not ascribable to the proliferation of mesangial cells like acute pyelonephritis, chronic pyelonephritis, minimal-change nephritic syndrome, chronic glomerulonephritis, renal amyloidosis, etc.

To evaluate renal functions according to the present invention, a biological specimen from an individual whose renal function should be tested is obtained and the concentration of megsin protein therein is measured following the above-mentioned methods. Preferably, the amount of megsin protein is determined from the measured concentration and the volume, and compared to that of a normal healthy person. To calculate the amount of megsin protein using urine as the sample, all urine of a day is pooled and the amount of urine is measured. Thereby, it is possible to determine the amount of megsin protein in a day's urine. Alternatively, it is possible to presume the amount by creatinine correction, even when the urine is sampled occasionally. The term "creatinine correction" refers to a method to correct the influence of dilution (or concentration) of the object component due to the changes in urine amount based on the creatinine concentration. The creatinine amount excreted daily into the urine is constant.

Based on this fact, the percentage occasionally collected urine occupies in the daily total excretion is calculated from the creatinine concentration, and the concentration of the object component in the same urine is converted into the total excretion of a day. Alternatively, correction methods generally used in the diagnosis of renal functions such as weight correction is applied to presume the amount in blood. Weight correction is a method, wherein the amount of blood component is calculated based on the volume presumed from the weight of the individual from whom the blood was taken.

Alternatively, changes in renal function can be also followed in a time course without having to convert into amounts by observing the changes of megsin protein concentration in a biological specimen derived from a certain individual. Alternatively, an abnormality in renal function can be determined by configuring the normal values for megsin protein concentration in body fluids of specific species or groups like the race, and then, comparing the megsin protein concentration (or amount) of an individual with the normal value.

The biological specimen used in the present invention can be urine or blood. Urine is more preferable since it can be collected in a noninvasive manner and is at the same time a sample that reflects the condition of renal functions directly. Although blood must be collected with some invasiveness, since megsin protein is a protein specific to the kidney, an abnormality of the measured value closely relates to an abnormality of renal function. Thus, a high specificity can be expected if the amount of megsin protein in blood and urine is used as indicators of renal function. Compared to the currently widely used method of screening for renal function disorder using urinary proteins as indicators, a more specific screening method with a superior sensibility can be expected by utilizing the megsin protein as the indicator. A method for evaluating renal functions by measuring the megsin protein in urine based on the immunological method for measuring the megsin protein using a monoclonal antibody will be explained in detail below.

(A) Preparation of the antibody

(1) Immunization of the animal and preparation of antibody-producing cells

The immunization of the animal is conducted, for example, as follows. A mammal, such as a rat, mouse, and so on, is immunized with the human megsin protein purified according to conventional methods (for example, T.Miyata et al., J.Clin.Invest., 120:828-836 (1998)). It is preferable to immunize animals of the same strain as the animal strain from which the permanently proliferating cell used during cell fusion is obtained. For example, mice at the age of 8 to 10 weeks are preferable. Mice of both genders will do. The immunization is conducted as follows: (1) the purified human megsin protein is mixed to an emulsion with an appropriate adjuvant (for example, Freund's complete adjuvant, aluminum hydroxide gel-pertussis vaccine, etc.), and (2) administered to the animal subcutaneously, intraperitoneally, intravenously, etc. Repeating this immunization process 2 to 5 times, with an interval of 1 to 2 weeks. The final immunization is accomplished by an intraperitoneal administration of 0.5 to 2 µg human megsin protein to the animal. Polyclonal antibodies are obtained from the body fluid of the thus immunized animal. 3 to 7 days after each immunization process, blood is collected from venus plexus of the eyeground, and the antibody titer of the serum is measured by the protein A rosette assay (Eur.J.Immunol., 4:500-507 (1974)) described below. The antibody or the antibody-producing cell is harvested as the antibody titer had elevated enough.

The protein A rosette assay is, for example, performed as follows: (1) 72 well Terasaki Plate (Falcon) is coated with the human erythroblast cell line K562 (Japanese Cancer Research Resources Bank (JCRS)); (2) samples diluted with PBS (sodium dihydrogen phosphate 2.90 g, potassium monohydrogen phosphate 0.20 g, sodium chloride 8 g, potassium chloride 0.2 g, distilled water 1 L) are added; (3) the plate is left standing for 30 minutes at 37°C in a CO₂ incubator; (4) the wells are washed with PBS; (5) sheep erythrocytes coated with protein A (Amersham Pharmacia Biotech) are added; and, (6) the formed rosettes are observed with a microscope.

Antibody-producing cells are harvested from the above human megsin protein immunized animal. Antibody-producing cells can be

obtained from the spleen, lymph nodes, peripheral blood, and so on. However, the spleen is especially preferred. For example, 3 to 4 days after the final immunization, the spleen is excised aseptically, cut in a Minimal Essential Medium (MEM) (Nissui Pharmaceutical), detach
5 tissues and cells with a tweezer, and after centrifugation at 1,200 rpm for 5 minutes the supernatant is discarded. Then the pellet is treated with Tris-HCl buffer (pH 7.65) for 1 to 2 minutes, and erythrocytes are eliminated. After washing 3 times with MEM, spleen cells used for cell fusion are obtained.

10 (2) Preparation of permanently proliferating cells

Any cell with permanent proliferation ability can be used as the permanently proliferating cell for the fusion. However, myeloma cells are generally used. It is preferable to use cells derived from the same specie as that from which the antibody-producing cell was
15 obtained. For example, in the case of mouse, the following cell lines are known as 8-azaguanine resistant mouse (BALB/c)-derived myeloma cell lines.

P3-X63Ag8-U1 (P3-U1) (Current Topics in Microbiol. Immunol., 81:1-7, (1978)),

20 P3/NS1/1-Ag4-1 (NS-1) (Eur. J. Immunol., 6:511-519, (1976)),

SP2/0-Ag14 (SP-2) (Nature, 276:269-270, (1978)),

P3-X63-Ag8653 (653) (J. Immunol., 123:1548-1550, (1979), and

P3-X63-Ag8 (X63) (Nature, 256:495-497, (1975))

These permanently proliferating cell lines are subcultured in
25 8-azaguanine media (normal media, RPMI-1640 medium supplemented with glutamine (1.5 mM), 2-mercaptoethanol (5×10^{-5} M), gentamicin (10 µg/mL) and fetal calf serum (FCS, CLS) (10%), supplemented with 8-azaguanine (15 µg/mL)), and passaged to normal media 3 to 4 days before cell fusion to ensure a cell count of 2×10^7 cells at the day
30 of cell fusion.

(3) Cell fusion

Fusion of cells is, for example, performed as follows. The antibody-producing cell obtained in (1), and the permanently proliferating cell prepared in (2) are mixed at a cell rate of 5 to
35 10:1 after washing well with MEM or PBS. After centrifugation at 1,200 rpm for 5 minutes, the supernatant is removed and the precipitated

cells are detached well. 0.1 to 1.0 mL/ 10^8 cells are added to a mixture of 1 to 4 g polyethylene glycol-1000 (PEG-1000), 1 to 4 mL MEM, and 0.5 to 1.0 mL dimethyl sulfoxide, and the mixture is kept at 37°C while stirring to fuse the cells. Then, the mixture is diluted by adding 3 mL MEM few times every 10 minutes to a total volume of 50 mL MEM to quench the cell fusion reaction. Next, the supernatant is discarded after centrifugation (1,500 X 5 min), cells are gently tapped, 100 mL normal medium (RPMI-1640 media, 10% FCS) is added, and the cells are suspended by gentle pipetting with a measuring pipette. 100 μ L suspension is dispensed to each well of a 96 well culture plate, and incubated at 37°C for 3 to 5 days in an 5% CO₂ incubator. 100 μ L of HAT medium (normal medium supplemented with hypoxanthine (10^{-4} M), thymidine (1.5×10^{-5} M), and aminopterin (4×10^{-7} M)) is added to each well of the culture plate, and incubated further for 3 days. Thereafter, half of the culture supernatant is discarded and the same amount of HAT medium is added thereto every three days. The incubation is continued for about 2 weeks at 37°C in a 5% CO₂ incubator.

(4) Screening and cloning of the hybridoma

Supernatants are removed from wells in which fusion cells are observed to grow in colonies, same amount of HT medium (HAT medium without aminopterin) is added, and the cells are incubated for 4 days. Part of the culture supernatant is harvested to measure the antibody titer against megsin protein by the aforementioned protein A rosette assay. The antibody titer can be also assayed by other methods like the following. For example, 1) by adding the hybridoma culture supernatant to a solid phase (e.g. microplate), where the megsin protein antigen is attached directly or together with a carrier; 2) adding anti-immunoglobulin antibody (if the cell for cell fusion is derived from mouse, anti-mouse immunoglobulin antibody is used) labeled with radioactive substances or enzymes, or protein A; and 3) detecting the anti-megsin protein monoclonal antibody bound to the solid phase. The antibody titer can be also assayed by: 1) adding the hybridoma culture supernatant to a solid phase, where an anti-immunoglobulin antibody or protein A is attached; 2) adding megsin protein labeled with radioactive substances or enzymes, and

3) detecting the anti-megsin protein monoclonal antibody bound to the solid phase.

Cloning is repeated 4 times by limiting dilution method for wells in which the production of antibodies that react to the megsin protein was observed, and cell lines, which show stable megsin protein antibody titers are selected as anti-megsin protein monoclonal antibody producing hybridoma cell lines.

(5) Preparation of the monoclonal antibody

The monoclonal antibody is produced by incubating the thus obtained hybridomas *in vitro* or *in vivo*. In case where the incubation is conducted *in vivo*, the hybridoma is transplanted to appropriate animals. It is preferable to use the same kind of animal as that from which the spleen cells for cell fusion were taken. For example, 2 to 4×10^6 cells of anti-megsin protein monoclonal antibody producing hybridoma cells obtained in (4) is administered intraperitoneally to each 8 to 10 week old BALB/c female mouse treated with pristane (reared for 2 weeks after intraperitoneal administration of 0.5 mL 2,6,10,14-tetramethylpentadecane-pristane). Subsequently, after 2 to 3 weeks, the abdomen of the mouse enlarges due to the accumulation of ascites, which contain the monoclonal antibody at a high concentration, in the visceral cavity of the mouse. The ascites are then collected from the mouse, centrifuged (3,000 rpm X 5 min) to remove solid matters, and the IgGs are purified. Alternatively, the hybridoma is cultured *in vitro* in a serum-free and an appropriate amount of antibody secretes to the supernatant. The ascites or the culture supernatant is salted out with 50% ammonium sulfate, and then dialyzed for 1 to 2 weeks with PBS. The dialyzed fraction is passed through a protein A Sepharose column, and the IgG fraction is collected to obtain a purified monoclonal antibody.

The isotype of the antibody is determined according to the Ouchterlony method (double immunodiffusion) ("Menekigaku jikken nyumon, Seibutsugaku jikkenhou 15" (A laboratory manual for immunology, Biochemical experimentation 15), Gakkai Shuppan Center, p74 (1981)). The quantity of the protein is calculated by the Folin method and by the absorbance at 280 nm ($1.4 \text{ (OD}_{280}) \approx \text{immunoglobulin 1 mg/mL}$).

(6) Characterization of the monoclonal antibody

The characterization of the monoclonal antibody obtained above can be performed by, for example, (1) immunoprecipitation method utilizing human lymphocyte derived cell lines, such as HSB-2, K562, and so on, the cell surface of which is labeled with iodine (J.Immunol., 138: 2850-3855 (1987)); (2) enzyme immunoassay (ELIZA) (J.Immunol., 142: 2743-2750 (1989)); and such.

(7) Preparation of labeled monoclonal antibody

The obtained purified monoclonal antibody may be enzyme labeled by methods like glutaraldehyde method (Immunochem., 6: 43 (1969)), periodic acid method (J.Histochem.Cytochem., 22: 1084 (1974)), maleimide method (J.Biochem., 79: 233 (1976)), pyridil disulfide method (Biochem.J., 173: 723 (1978)), etc.

For example, the periodic acid method is performed as follows:

(1) periodic acid (38.5 mg/mL) is added to the peroxidase solution (4 mg/mL) while stirring; (2) reacted at room temperature for 20 minutes; (3) the buffer is exchanged using PD-10 (Amersham Pharmacia Biotech) substituted with 1 mM acetate buffer (pH 4.5); (4) 40 μ L of 0.2 M sodium hydroxide is added; (5) 10 mg of monoclonal antibody dialyzed with 10 mM carbonate buffer is added (pH 9.5); (6) react at room temperature for 2 hours; (7) after the reaction is completed, the mixture is cooled on ice; (8) 100 μ L solution of sodium boron hydride (4 mg/mL) is added and reacted for 2 hours; (9) the reaction solution is changed to PBS using PD-10; (10) centrifuged at 3,000 rpm for 30 minutes; (11) the supernatant is gel filtrated using Sephacryl S200HR26 X 30 (Amersham Pharmacia Biotech); (12) the labeled monoclonal antibody fraction is obtained by detecting the absorbance at 403 and 280 nm; (13) bovine serum albumin (10 mg/mL) is added to the obtained fraction; (14) stored at -20°C, and diluted with PBS-Tween (Trademark) 20 before usage.

(B) Preparation of granules for detection

The granules for detection used herein may be prepared by physically or chemically binding anti-megsin protein monoclonal antibody to appropriate granules, for example, gel for chromatography. The method used in the present invention for binding the antibody to chemically activated granules is a preferable method due to the expected binding stability. More specifically, the method for

binding the antibody used in the present invention to granules that are actively tosylated by p-toluenesulfonyl chloride can be given.

Granules to be used are made of glass, agarose, Sepharose, agarose filled porous diatomite, hydrophilic copolymerized acrylic gel, polystyrene, and so on. Use of magnetic granules enables collection of the granules with the use of magnet, and so on, thereby allowing detection of small quantity samples. For example, by placing substances capable of being magnetic (e.g. Fe_2O_3) in the core of the granules, it is possible to make the granules superparamagnetic.

Such granules are commercially available as solid phases for immunological analyses. The granule may take any appropriate form such as a sphere, amorphous fracture, etc., however, sphere shaped granules are preferred. There is no limitation on the particle size of the granule, and an average particle size, for example, of 5 to 1000 μm can be given. Furthermore, the use of granules with larger specific gravity than that (about 1) of the reaction solution facilitates collection of the granules, giving an effect similar to that when magnetic granules are used. Furthermore, the use of granules with larger specific gravity is more advantageous for antibodies whose binding is easily disassociated, because gentle centrifugation conditions are possible when collecting the granules.

Anti-megsin protein antibodies can be bound to the granules not only by direct binding but also by indirect binding. For example, if a mouse monoclonal antibody is used as the antibody, an antibody that recognizes mouse IgG is attached to the granule and the mouse antibody can be bound to the granule indirectly. Such antibodies are called secondary antibodies. Indirect binding can be also accomplished by utilizing protein A or protein G, which bind to the constant region of immunoglobulin, or by capturing avidin-attached granules with biotinylated antibodies, and so on. To chemically bind the secondary antibody, protein A, or protein G to the granule, it is preferable to activate the granules before binding. As an activation method of the granule, any appropriate activation method used to conjugate proteins to the granule can be selected. Such activation methods include, the tosyl chloride method, bromcyane method, bromacetyl method, glutaraldehyde method, etc. There are

also commercially available activated granules. Activation and binding of these activated granules to proteins, such as secondary antibodies, protein A, protein G, and such, can be performed according to conventional methods.

5 In addition, granules conjugated to secondary antibodies, protein A, protein G, and so on are also commercially available. Granules mentioned below are known as commercially available granules.

10 Dynabeads™ M-450, M-280 provided by Veritas, and imported by Dynal Japan

Sheep anti-mouse IgG coated type

Goat anti-mouse IgG coated type

Sheep anti-rat IgG coated type

Sheep anti-rabbit IgG coated type

15 Polyscience Inc.

Goat anti-mouse IgG (H&L) carboxylate beads

Goat anti-rabbit IgG (H&L) carboxylate beads

Protein A carboxylate beads

Goat anti-rabbit IgG (H&L) micromagnet particle

20 Goat anti-rabbit IgG (H&L) micromagnet particle

Protein A micromagnet particle

Sheep anti-mouse IgG (H&L) micromagnet particle

25 The binding of the present antibody to the granules above is conducted as follows: (1) unspecific binding is prevented by treating the granules suspended in appropriate media with a protein solution, and (2) mixing the ascites containing the antibody or the purified antibody solution.

(C) Detection method

30 The detection method of the present invention is accomplished by collecting blood or urine from the subject, and using the supernatant after centrifugation as the specimen. The centrifugation of urine is done to separate the precipitate, and the supernatant of urine left standing still, or urine from which the precipitate is removed by filtration can be also used. Specimen
35 diluted with the detection granule prepared above and the labeled antibody obtained in (7) are mixed, and then, incubated at room

temperature for 2 hours. After the reaction has come to an end, the mixture is washed, and the substrate solution is added for chromogenic reaction. Then, the mixture is centrifuged to remove the granules, the supernatant is transferred onto a microplate, and the absorbance is measured. A specimen from a normal healthy person is also assayed according to the same method, and the absorbance is compared. Mere comparison of the concentration of the megsin protein, as well as a comparison of the absolute quantity of megsin protein in the body fluid, which is obtained by multiplying with the volume of body fluid of the individual, or comparisons based on of other similar corrected values might be performed.

(D) Kit

The materials used to perform the experiment above can be supplied as a kit. The kit may comprise a detection granule, to which the antibody is conjugated as described above, and a magnet. It may also include an antibody labeled with a marker molecule. Further, the kit of the present invention may include test tubes, centrifugation tubes, other similar containers, pipettes or other suction instruments, and microscopes. Furthermore, enzyme substrates necessary for detecting the label, and positive or negative standard samples may be combined. Solid granules and antibodies, which are the raw material for the detection granules mentioned above, may be included instead of the detection granules. The invention is described in detail with reference to the examples below, but it is not to be construed as being limited thereto.

Brief Description of the Drawings

Figure 1 shows the result of Western blotting using a polyclonal antibody specific to synthetic peptide-2, which has a partial amino acid sequence of megsin protein (SEQ ID NO: 12), as the antigen. Each lane represents the following:

- 1: MBP
- 2: MBP-megsin protein
- 3: His-megsin protein
- 4: MBP-PAI II
- 5: megsin protein expressed in CHO cells

6: body fluid of the silkworm infected with a recombinant virus
transfected with the megsin protein gene

7: body fluid of the silkworm infected with a recombinant virus
without transfection by megsin protein gene

5 8: body fluid of the virus-uninfected silkworm

9: normal human serum

Figure 2 shows the result of Western blotting using a polyclonal
antibody specific to synthetic peptide-3, which has a partial amino
acid sequence of megsin protein (SEQ ID NO: 13), as the antigen. Each
10 lane represents the same proteins as in Figure 1.

Figure 3 shows the result of Western blotting using a polyclonal
antibody specific to synthetic peptide-342, which has a partial amino
acid sequence of megsin protein (SEQ ID NO: 15), as the antigen. Each
lane represents the same proteins as in Figure 1.

15 Figure 4 shows the result of Western blotting using a polyclonal
antibody specific to MBP- megsin protein, as the antigen. Each lane
represents the same proteins as in Figure 1.

Figure 5 is a graph showing assay results of urinary megsin
proteins by ELISA. The vertical axis shows assayed value of megsin
20 protein (creatinine ratio: AU). Urine from normal healthy person (16
samples), from patients with IgA nephropathy (24 samples), and from
patients with minimal-change nephritic syndrome (24 samples) was used
as specimens.

Figure 6 shows the assay result of Western blotting on urinary
25 megsin protein. Each lane represents the following:

1: MBP-megsin protein

2 to 5: concentrated urine from normal healthy persons

6 and 7: concentrated urine from patients with IgA nephropathy

Figure 7 shows a microscopic photograph of immunostained kidney
30 tissue of patients with IgA nephropathy.

Figure 8 shows a microscopic photograph of immunostained human
kidney tissue of a normal person. I is a magnification of 33 times,
and II to IV is of 80 times.

Figure 9 shows a microscopic photograph of immunostained human
35 kidney, liver, and spleen tissue of normal healthy person. V is a
photograph with a magnification of 33 times, VI is of 80 times, and

VII and VIII are of 40 times.

Figure 10 shows a microscopic photograph of immunostained rat kidney, liver, and spleen tissue of normal rat using rat megsin peptide-2 antibody. I and II are photographs with a magnification
5 of 100 times, and III and IV are of 40 times.

Figure 11 shows the detection result of megsin protein by immunoprecipitation.

Lane 1: anti-megsin peptide-2 antibody(-)

Lane 2: anti-megsin peptide-2 antibody(+)

10 Lane 3: molecular marker

Figure 12 shows the ELISA result using CHO-megsin or MBP-megsin as the antigen and anti-megsin peptide-2 antibody. The vertical axis shows the absorbance at 492 nm, and the horizontal axis shows the antigen concentration ($\mu\text{g/mL}$).

15 Figure 13 shows the ELISA result using CHO-megsin or MBP-megsin as the antigen and anti-megsin peptide-4 (257-272) antibody. The vertical axis shows the absorbance at 492 nm, and the horizontal axis shows the antigen concentration ($\mu\text{g/mL}$).

20 Best Mode for Carrying out the Invention

[Example 1] Mesangial cells

Human mesangial cells and CHO (Chinese hamster ovary) cells obtained from TaKaRa (Tokyo, Japan) were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum at 37°C in
25 a 5% CO₂ incubator. Human mesangial cells were subcultured for 7 to 10 generations, and then used for the following experiments.

[Example 2] Production of polyclonal antibodies against synthetic peptides of the megsin protein

30 Polyclonal antibodies against megsin protein were produced by using regions having low identity with other members of the serpin family and which are hydrophilic as the immunogens. More specifically, polypeptides with the following amino acid sequences derived from the amino acid sequence of human megsin (SEQ ID NO: 2)
35 and rat megsin (SEQ ID NO: 19) were selected:

Position from	Amino acid sequence	SEQ ID NO/No.
N-terminus		
16- 30	FREMDDNQGNVFF	11/1
72- 86	SQSQLSQLKRVFSD	12/2
339-354	ATGSNIVEKQLPQSTL	13/3
257-272	NLMEWTNPRRMTSKYV	14/4
342-356	SNIVEKQLPQSTLFR	15/342
74- 87	LGLQYQLKRVLAD	16/rat1
341-354	ESNIVEKLLPESTV	17/rat2

Peptides having the amino acid sequences shown above with a C attached to the N-terminus (for peptide 3 at its C-terminus) were synthesized on the automatic synthesizer model 432A (Perkin Elmer, Foster City, CA). After purifying the synthetic peptide by reverse phase HPLC, the peptide was freeze-dried and used in a competitive experiment to confirm immunity and immunological specificity.

Each of these synthetic peptides were bound to bovine thyroglobulin (Sigma) using N-(6-maleimidocaproyloxy)succinimide (Dojindo Molecular Technologies Inc.), were dialyzed against 0.85% NaCl solution, and were mixed well with the adjuvant (Difco) to emulsion, and were administered subcutaneously to a rabbit. 3 weeks after priming (20 µg/rabbit), second immunization (50 µg/rabbit) was carried out, and thereafter, four immunizations (50, 100, 200 µg/rabbit) were performed every two weeks. Freund's complete adjuvant was used for the priming, and thereafter, incomplete Freund's adjuvant was used. After 41 days and 55 days, the antibody titer of the serum was evaluated by enzyme-linked immunosorbent assay (ELISA) to confirm that the serum obtained by blood sampling reacts with synthetic peptide. To conduct primary reaction, 100 µL of continuously diluted antiserum was added to each well of the 96 well plate, in which 50 ng/well antigen had been immobilized. After washing, the HRP conjugated goat anti-rabbit IgG (Cappel) was reacted against it in the second reaction. After washing, the coloring reaction was done using orthophenylenediamine (Wako Pure Chemical Industries, Ltd.) as the substrate and absorbance was measured at 492 nm. An increase in antibody titer was confirmed.

[Example 3] Purification of polyclonal antibodies against synthetic peptides of megsin protein

The polyclonal antibodies against each synthetic peptide of megsin protein were purified by immunoaffinity chromatography according to conventional methods (Cell Engineering supplement "Jikken Protocol Series Kou-peptide Jikken protocol (Experimental protocol series: Anti-peptide experimental protocol)", Shujun-sha, Japan). More specifically, the method was carried out as follows:

- 1) an affinity column was prepared by immobilizing each synthetic peptide to FMP (2-fluoro-1-methylpyridinium toluene-4-sulfonate) activated Cellulofine (Seikagaku Corp.);
- 2) after the rabbit serum showing increased antibody titer was diluted with PBS (-), the antibody was purified by affinity using the peptide column.

It was demonstrated that the obtained purified antibody is specific to the megsin protein, by confirming its reaction against megsin protein fusion protein by Western blotting (Figure 1 to 4).

[Example 4] Preparation of antibodies against rat megsin synthetic peptides

Each rat megsin synthetic peptide was bound to keyhole limpet hemocyanin (KLH) (Calbiochem-Novabiochem, La-Jolla, CA). Namely, KLH suspended in 0.05 M sodium phosphate buffer (pH 7.2) was reacted with m-maleimidobenzoyl-N-hydroxysuccinimidoester (MBS) diluted in dimethylformamide by stirring 30 minutes at room temperature. The reactant was dialyzed with 0.05 M sodium phosphate buffer (pH 6.0) and was used as the KLH-MB. Then, the obtained KLH-MB was incubated with the above-mentioned peptide suspended in distilled water. Half of 0.2 M disodium hydrogenphosphate was added to KLH-MB and was stirred for 3 hours at room temperature. Following the reaction, it was dialyzed with sodium phosphate buffer and was finally freeze-dried. The rabbit was immunized with the obtained KLH conjugated synthetic peptide and the adjuvant. Freund's complete adjuvant (FCA) (DIFCO Laboratories, USA) was used for the priming, and Freund's incomplete adjuvant (DIFCO Laboratories, USA) was used for the immunization that followed. Immunization was conducted 5 times with 2 to 3 weeks

intervals. IgG was purified from antiserum using Protein A affinity column (Pharmacia Biotech, Uppsala, Sweden). Serum was also obtained from rabbits before immunization, and the IgG was purified in the same manner.

5

[Example 5] Studies on reactivity of the rabbit polyclonal anti-megsin peptide IgG

10 The reactivity of the rabbit IgG, which was produced using megsin protein as the immunogen, was studied using the following various proteins as antigens. MBP-megsin protein and His-megsin protein were prepared as follows: 1) the coding region was amplified by PCR based on the nucleotide sequence of megsin gene described in SEQ ID NO: 1; 2) the amplified products were inserted into maltose binding protein fusion protein expression vector pMAL-c (New England Biolab) to obtain vectors that express the fusion protein of MBP and megsin protein; 3) E.coli were transformed with this vector; and then, 15 4) the MBP-megsin protein was obtained by purifying the expression product from the disrupted cell solution by affinity chromatography utilizing amylose, and such. To obtain His-megsin protein, a fusion protein expression vector containing aforementioned megsin gene in the vector ptrcHisA (Invitrogen), which expresses the inserted gene as a fusion protein with a histidine tag, was constructed. This vector was transformed into E.coli JM109, and following the induction of expression by IPTG, the His-megsin protein was obtained as a fusion 20 protein purified by nickel column and such from the disrupted cell solution.

25 Then, recombinant megsin was expressed in CHO cells as follows. The c-myc tag and histidine tag was ligated to the N-terminus of the full-length coding region of megsin cDNA by the induction of mutation utilizing PCR. The tag conjugated megsin cDNA was cloned into 30 mammalian cell expression vector pREP9 (Invitrogen). PREP9 containing human megsin cDNA was transformed into CHO cells (10^5 cells), which were cultured on a 6 well plate at 37°C in a 5% CO₂ incubator, using LipofectAMINE (GIBCO BRL, Gaithersburg, MD). Stable transformants were selected with 0.5 mg/ml geneticin disulfate (Wako 35 Pure Chemical Industries). Then, the c-myc-histidine tag conjugated

megsin recombinant (CHO megsin) was purified from the culture supernatant by (His)6 affinity column.

The megsin protein(+)virus-infected silkworm body fluids refers to body fluids of a silkworm, which is infected with megsin gene introduced virus according to known methods (J.General Virology 78: 3073-3080 (1979)). On the other hand, megsin protein(-)virus-infected silkworm body fluids refers to body fluids of a silkworm, which is infected with a virus without the megsin gene. The gene encoding megsin necessary for designing these megsin recombinants can be synthesized by PCR using mRNA of the mesangial cell as the template, or obtained from the megsin gene cloning vector (Accession No. FERM BP-6518) constructed by the inventors.

Reactivity against plasminogen activator inhibitor-1 (PAI I), MBP-plasminogen activator inhibitor-2 fusion protein (MBP-PAI II), and so on was also observed. These proteins were selected to confirm the cross-reactivity of the antibody, and share some extent of homology with the megsin protein.

MBP-megsin protein,

His-megsin protein,

MBP-PAI II,

PAI I,

megsin protein expressed in CHO cells,

megsin protein (+) virus-infected silkworm body fluid,

megsin protein (-)virus-infected silkworm body fluid,

non-virus-infected silkworm body fluid, and

human serum albumin.

Each protein solution was treated with same amount of sample buffer (0.25% Tris-HCl, 2% SDS, 30% glycerin, 10% β -mercaptoethanol, 0.025% Bromophenol Blue) (Daiichi Pure Chemicals), heated at 100°C for 5 minutes, and the proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, U.K., Nature 227: 680-685 (1970)). The proteins separated by SDS-PAGE was blotted on polyvinylidene difluoride (PVDF) membrane (BioRad) with a constant voltage of 100 V for 1 hour, using the blotting solution (25mM Tris-HCl, 192 mM glycine, 20% methanol, pH 8.3). Following the washing of the blotted PVDF membrane with distilled water, the membrane was blocked

for 3 hours in TTBS solution containing 5% Block Ace. After washing the PVDF membrane with TTBS (20 mM Tris, 500 mM NaCl, 0.05% Tween20, pH 7.5), it was reacted at 4°C, overnight with the primary antibody, rabbit polyclonal anti-megsin peptide IgG, diluted in TTBS. Then it
5 was detected with Amplified Alkaline Phosphatase Immuneblot Kit (BioRad). That is, it was first incubated for 1 hour with TTBS diluted biotinylated goat anti-rabbit IgG at room temperature, then reacted with streptavidin - biotinylated alkaline phosphatase complex, which was prepared in advance by incubating streptavidin and biotinylated
10 alkaline phosphatase for 1 hour at room temperature. The PVDF membrane was washed in TTBS, and then incubated with the substrate (nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate, p-toluidine salt solution) for about 30 minutes at room temperature to visualize the antibody bound to the primary antibody. The reaction
15 was quenched by reacting with a sufficient amount of distilled water.

The results are shown in Figures 1 to 4. The antibodies reacted in each figure were obtained using the following substances as the immunogen.

- Figure 1. immunogen: megsin peptide-2
- 20 Figure 2. immunogen: megsin peptide-3
- Figure 3. immunogen: megsin peptide-342, and
- Figure 4. immunogen: MBP-megsin protein

[Example 6] Enzyme labeling of affinity purified anti-megsin peptide
25 IgG

The affinity purified anti-megsin peptide IgG obtained in Example 3 was labeled with alkaline phosphatase using a commercial labeling kit (AP labeling kit: Boehringer-Mannheim) following the instructions in the kit. Biotinylation was performed with another
30 labeling kit (ECL protein biotinylation module: Amersham Pharmacia Biotech) following the instructions.

[Example 7] Measurement of urinary megsin protein by ELISA

Urine from 16 normal healthy persons, 24 IgA nephropathy
35 patients, and 24 minimal-change nephritic syndrome patients were centrifuged to exclude the insoluble material. This was then

concentrated using an ultrafilter, with a molecular weight cut-off of 5,000 (Ultrarafree, Millipore). Each well of the 96 well ELISA plate was coated with rabbit polyclonal anti-megsin peptide-2 IgG. 120 μ L PBS (-) was poured into each well, then 120 μ L concentrated urine was added thereto and was left standing overnight at 4°C. The wells were washed with PBS (-), and Block Ace (Dainippon Pharmaceutical) was added to conduct blocking for 2 hours at room temperature. The wells were then washed with PBS (-) containing 0.05%(w/v) Tween20 (Wako Pure Chemical), alkaline phosphatase labeled rabbit polyclonal anti-megsin peptide-1 antibodies were added, and left standing for an hour at room temperature. After washing with Tween-PBS, 100 μ L of coloring substrate solution (1 mg/mL orthophenylenediamine (Wako Pure Chemical) - 1 M diethanolamine (Wako Pure Chemical) buffer (pH 9.8)) was added to each well. After reacting at room temperature, the reaction was quenched with 50 μ L/well 1 N sodium hydroxide, and the absorbance at 405nm was measured with a microplate reader (Spectra MAX 250, Molecular Devices). The megsin protein concentration in the urine was determined from the calibration curve. The creatinine concentration in the same urine specimen was determined with the commercially available creatinine-measuring reagent (measuring reagent for automated analyzer "Dia"-Crea, Dia Pharmaceutical), and the amount of megsin protein was corrected as creatinine ratio. The result is shown in Figure 5. A significant difference in megsin concentration was observed between that of the normal healthy persons and patients of IgA nephropathy ($p<0.001$), or between that of IgA nephropathy patients and minimal-change nephritic syndrome patients ($p<0.001$).

Since a significant difference between normal healthy persons and IgA nephropathy patients was observed, onset of renal disorders could be confirmed by measuring urinal megsin protein. Furthermore, due to the significant difference observed between patients of IgA nephropathy and minimal-change nephritic syndrome, it was revealed that these diseases could be distinguished by measuring urinal megsin protein according to the present invention. These diseases cannot be distinguished by conventional screening methods using urinary protein as indicators, because positive results would be shown for

both diseases.

Furthermore, in comparisons of urinary megsin protein concentration without creatinine correction, although megsin protein concentration tends to be higher for patients with renal disorders, it seemed difficult to prove a statistically significant difference with a small number of examples. Thus, it is preferable to compare the amounts by methods like creatinine correction and such, when implementing the measurement of megsin protein in urine according to the present invention.

[Example 8] Detection and assay of urinary megsin protein by Western blotting

Urine from 8 normal healthy persons and 9 IgA nephropathy patients were centrifuged, after discarding insoluble material, the solution was concentrated with an ultrafilter with a molecular weight cut-off of 5,000 (Ultrafree, Millipore). The concentrated urine was treated with same amount of sample buffer (0.25% Tris-HCl, 2% SDS, 30% glycerin, 10% β -mercaptoethanol, 0.025% Bromophenol Blue) (Daiichi Pure Chemicals), heated at 100°C for 5 minutes, and separated in a gradient gel with a gel gradient of 4 to 20% (Daiichi Pure Chemicals) by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, U.K. (1970) Nature 227, 680-685). The protein separated by SDS-PAGE was blotted on poly vinylidene difluoride (PVDF) membrane (BioRad) with constant voltage of 100 V for 1 hour, using the blotting solution (25 mM Tris-HCl, 192 mM glycine, 20% methanol, pH 8.3). Following the washing of the blotted PVDF membrane with distilled water, the membrane was blocked for 3 hours in TTBS solution containing 5% Block Ace. After washing the PVDF membrane with TTBS (20 mM Tris, 500 mM NaCl, 0.05% Tween20, pH 7.5), it was reacted overnight at 4°C with a primary antibody solution, rabbit polyclonal anti-megsin peptide IgG diluted in TTBS.

Then it was detected with Amplified Alkaline Phosphatase Immuneblot Kit (BioRad). That is, it was first incubated for 1 hour with biotinylated goat anti-rabbit IgG diluted with TTBS at room temperature, then reacted with streptavidin-biotinylated alkaline phosphatase complex, which was prepared in advance by incubating

streptavidin and biotinylated alkaline phosphatase for 1 hour at room temperature. The PVDF membrane was washed in TTBS, and then incubated with the substrate (nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate, p-toluidine salt solution) for about 30 minutes at room temperature to visualize the antibody bound to the primary antibody. The reaction was quenched by reacting with a sufficient amount of distilled water. The visualized bands were quantitatively analyzed using Densitograph gel documentation system (AE-6920M-05, Atto) and densitograph analyzing software (AE-6920WSE/MSE, Atto). The result (electrophoretogram) is shown in Figure 6. The measurements of the image analyzer are as follows. Dense bands were observed for IgA nephropathy patients compared to the bands of concentrated urine of normal healthy persons. It was revealed that quantitative measurement of the megsin protein is possible by quantitatively analyzing the density of these bands. Measurement result of the image analyzer

Lane	Measurement result
1	Molecular weight marker
2	8.5
3	5.2
4	13.8
5	4.8
6	32.8
7	15.1

[Example 9] Preparation of granules for detection

The granules for detection of the present invention were produced by conjugating anti-megsin peptide IgG to commercially available magnetic granules through anti-mouse IgG antibody. Dynabeads™ M-450 tosylactivated (homogenous tosyl activated paramagnetic polystyrene granules: Dynal Biotech) was used as the granules of the present invention, and a 1mM hydrochloric acid solution thereof was washed once with sterile distilled water and then with 0.2 M boric acid buffer (pH9.5). Then, anti-megsin peptide IgG antibody was dissolved in 0.2 M boric acid buffer (pH 9.5) at a concentration of 150 µg/mL, and this solution was added to an equal

amount of the activated granule suspension (antibody/granule=75 μ g/15mg). Then, the mixture was incubated for 24 hours at 22°C with gentle stirring. The antibody-conjugated granules were collected with a magnet, and the supernatant was discarded leaving the granules

5 attached to the magnet. Then the granules were washed as follows:

(1) with 5 mL 0.1 M PBS (pH7.0) for 10 minutes,

(2) with 5 mL 1 M ethanolamine-HCl (pH 9.5) for 2 hours,

(3) with 5 mL 0.05 M Tris (pH 7.5) containing 0.1 M sodium chloride, 0.1% bovine serum albumin (BSA), and 0.1% Tween20 for 12 hours, and

10 (4) with 5 mL buffer of (3) without Tween20 for 2 hours.

Thereafter, granules were collected with a magnet, supernatant was discarded, and the granules were suspended in PBS (-)/BSA containing

10% Block Ace at a concentration of about 4×10^8 granules/mL (30 mg/mL).

Thus obtained IgG-conjugated granules are stable for at least 6 months
15 at 4°C.

[Example 10] Measurement of urinary megsin protein by the magnetic granule method

Megsin protein concentration of occasional urine samples from
20 12 normal healthy persons and 24 patients with different renal diseases were measured by the magnetic granule method utilizing antibody-conjugated granules prepared in Example 9. 12 of the renal disease patients were affected with IgA nephropathy, and the other 12 were affected with minimal-change nephritic syndrome. The urinary
25 samples were kept at -80°C, and were thawed before measurement. They were centrifuged at 3,000 rpm for 5 minutes, and the supernatant thereof were used as the sample. The measurement was carried out as follows:

500,000 magnetic granules conjugated to the primary antibody
30 (rabbit polyclonal anti-megsin peptide-2 antibody) were put into a tube (1.5 mL) blocked beforehand with Block Ace (Dainippon Pharmaceutical). 500 μ L of each urinary sample and equal amounts of alkaline phosphatase labeled rabbit polyclonal anti-megsin peptide-1 antibodies dialyzed with PBS (-) were mixed, and reacted for 2 hours
35 at room temperature while rotating. Then, after centrifugation at 3,000 rpm for 5 minutes, leaving the granules attached to the magnet,

the supernatant was discarded, and 1mL washing solution(0.05% (w/v) Tween 20 (Wako Pure Chemical) containing PBS (-) (pH7.4) (Tween-PBS)) was added and the granules were washed while stirring. The washing was repeated 4 times, and then, 100 µL substrate solution was added to react for 20 to 30 minutes with stirring. 1 M diethanolamine (Wako Pure Chemical) buffer (pH 9.8) containing 1 mg/mL orthophenylenediamine (Wako Pure Chemical) was used as the substrate solution. Following the completion of the reaction, the reaction was quenched with 50 µL 1 N sodium hydroxide, and absorbance at 405 nm was measured. The urinary megsin protein concentration was calculated from the calibration curve. Creatinine concentration of the same urinary sample was measured as in Example 7, and the megsin protein concentration was corrected as creatinine ratio.

According to the result of the measurement, urinary megsin protein measurement values (creatinine correction value: AU) of IgA nephropathy patients were distributed in the top region compared to those of the normal healthy person. Additionally, the urinary megsin protein measurement values of IgA nephropathy patients were also higher than those of minimal-change nephritic syndrome patients. Thus, according to the present invention, it is possible not only to decide whether a person is affected by a renal disorder, but definitely identify the state of the renal disorder.

[Example 11] Immunohistochemical staining of renal tissue using megsin peptide antibody

Renal tissues were taken from 3 IgA nephropathy patients (2 male, 1 female: age 21 to 48 years old) with their permission. Tumor-less tissues taken from surgically excised renal tissues from patients with renal tumors, were used as normal renal tissue specimens. No abnormality was observed in urine of the patient who provided the normal renal tissue, and from a histological point of view, those who showed an abnormality in glomus were excluded. Tissues from organs other than kidneys, were also obtained as controls from patients with their permission. Rat kidney and other organs were obtained from normal Wister rats (male, 8 weeks old).

The renal tissue was embedded in frozen tissue embedding agent

(O.C.T.compound) according to the conventional method. 4 μ m frozen sections were prepared from the frozen embedded tissues using cryostat. Thus prepared frozen sections were mounted on a slide coated with 3-aminopropylethoxysilane (Sigma) (immobilization with 4% paraformaldehyde, 15 minutes).

The frozen sections were washed with PBS containing 0.5% Tween 20, blocked with 4% skim milk, and incubated overnight with anti-megsin peptide-2 antibody and anti-megsin peptide-4 antibody in a humidification chamber at 4°C. Tissue sections were washed and incubated at room temperature for 2 hours with peroxidase labeled goat anti-rabbit IgG antibody (DAKO) diluted to 1:100. 3,3'-diaminobenzidine solution containing 0.003% oxygenated water was used for the detection of peroxidase. The nuclei were stained with hematoxylin. Hematoxylin/eosin staining was performed following conventional methods.

The micrograph (ECLIPSE E400: magnification of 80 diameters, Nikon) of the immunohistochemically stained renal tissue of the IgA nephropathy patient is shown in Figure 7. As is apparent from the picture, the human renal glomeruli tissue has a site that is stained with the peptide antibody of the present invention. Particularly, a notable positive staining was observed inside the mesangial cell and mesangial matrix, but the urinary tubule was not stained.

The glomerulus of human renal tissue was stained with human megsin peptide-2 antibody (Figure 8-I and 8-II), or with human megsin peptide-4 antibody (Figure 9-V and 9-VI). Particularly, notable positive staining was observed in the mesangium region. On the other hand, interstitis of the renal tissue was not stained by human megsin peptide-4 antibody (Figure 8-I). The hepatic tissue (Figure 9-VII), spleen tissue (Figure 9-VIII), pancreatic tissue, heart tissue, and aortic tissue were also not stained with human megsin peptide-2 antibody (data not shown). Specificity of the immunostaining could be confirmed, since excess megsin peptide-2 inhibited staining of the tissues completely (Figure 8-IV). No immune reaction could be detected with rabbit IgG of rabbits before immunization (Figure 8-III). Further, specific immunostaining could not be conducted on glomerulus of human renal tissue with antibodies against megsin peptide-1 or

megsin peptide-3 in comparison to megsin peptide-2 antibodies and megsin peptide-4 antibodies (data not shown).

Positive reactions were observed also in glomerulus of rat renal tissues by immunostaining with megsin proteins (rat megsin peptide-2; Figure 10-I). No reaction was observed in renal stroma, hepatic tissue (Figure 10-III), or spleen tissue (Figure 10-IV). Immunological reactions against rat renal tissue were arrested by incubating beforehand with rat megsin peptide-2 (Figure 10-II). No Immunological reaction was detected with rabbit IgG of rabbits before immunization, or with antibodies against rat megsin peptide-1 (data not shown).

[Example 12] Immunoprecipitation

An attempt was made to detect the megsin protein to confirm the existence of megsin protein in human mesangial cells. Namely, immunoprecipitation utilizing [³⁵S] methionine (T.Minori. et al., J.Biol.Chem. 259: 560 (1984)) was conducted, and thus obtained immune complex were analyzed by SDS-PAGE and autoradiography.

First, all protein elements were labeled by incubating mesangial cells in the labeling media (DMEM containing 10% heat inactivated fetal calf serum and [³⁵S] methionine (3.7 Mbq/mL)) at 37°C for 16 hours in a 5% CO₂ incubator.

Then cells were washed with cold PBS, and suspended with 200 µL of TSA (Tris-HCl buffer (pH 8.0), 0.14 M Sodium chloride, 0.025% sodium azide) containing 1% Triton X-100, 1% bovine hemoglobin, 1 mM iodoacetamide, 0.2 U/mL aprotinin, and 1 mM PMSF (phenylmethanesulfonyl fluoride). After left standing at 4°C for 1 hour, the solution was centrifuged at 100,000 X g, 4°C for 1 hour to obtain the lysate.

The radiolabeled antigen (10⁵-10⁶ cpm) and 1 µL anti-megsin peptide-2 polyclonal antibody (0.7 mg/mL) were incubated at 4°C for 90 minutes.

It was further incubated at 4°C for 90 minutes after adding protein G-sepharose (Pharmacia Biotech) to precipitate the immune complex. The precipitate was washed 3 times with TSA containing 0.1% Triton X-100 and 0.1% bovine hemoglobin, and once with 0.05 M Tris-HCl

buffer (pH 6.8).

The washed immune precipitate was suspended in 30 μ L sample buffer (0.5 M Tris-HCl buffer (pH 6.8), 10% SDS, β -mercaptoethanol, glycerol, 1% BPB), and then was boiled for 5 minutes. After mixing with a vortex mixer to suspension, the mixture was centrifuged at 200 X g for one minute. The supernatant was analyzed by SDS electrophoresis and autoradiography according to a conventional method. A 14 C-methylated protein mixture (M.W. 220, 97.4, 66,46, 30 kDa; Amersham International) was used as the molecular weight marker. The result is shown in Figure 11. The polypeptide, which is specifically recognized by anti-megsin peptide-2 antibody, was observed at around M.W. 50 kDa (the band indicated "meg" in lane 2).

[Example 13] Measurement of megsin by ELISA

ELISA assay was conducted on megsin protein expressed in CHO cells (CHO-megsin protein: T.Miyata et al., J.Clin.Invest. 120: 828-836 (1998); PCT/JP98/04269) and MBP-megsin protein described in Example 5.

Purified CHO-megsin protein solution (50 μ L/well) diluted stepwise with 50 mM carbonate buffer (pH 9.6) was added to a 96 well ELISA plate, and was left standing at 4°C overnight. 350 μ L Block Ace (Dainippon Pharmaceutical) was added to each well after washing with PBS, and blocking was done at room temperature for 2 hours. Then, the wells are washed with PBS containing 0.05% Tween 20 (Tween-PBS). 50 μ L of anti-megsin peptide-2 polyclonal antibody and anti-megsin peptide-4 polyclonal antibody obtained in Example 2 and 3, which were diluted in Tween-PBS, was added to each well, and the plate was left standing at room temperature for 1 hour. The wells were washed with Tween-PBS and biotinylated anti-rabbit IgG (Cappel) was added to the wells, and the plate was left standing at 4°C overnight, and washed with Tween-PBS.

Peroxidase-labeled streptavidin solution (Amersham) was added to each well and left to stand for an hour at room temperature. The wells were again washed with Tween-PBS, and 100 μ L/well orthophenylenediamine coloring substrate solution (orthophenylenediamine dissolved to a concentration of 0.04% in

citric acid-phosphate buffer (pH 5.0) containing 0.009% oxygenated water) was added to each well. After reacting for 10 to 30 minutes at room temperature in the dark, 50 μ L of 2 M sulfuric acid was added to each well to quench the reaction, and the absorbance at 492 nm was measured with a microplate reader (SPECTRAMax250, Molecular Device, Japan).

The same was performed with MBP-megsin protein. The results are shown in Figure 12 (megsin peptide-2 antibody) and Figure 13 (megsin peptide-4 antibody). The results revealed that both peptide-2 antibody and peptide-4 antibody react specifically with megsin protein in a concentration dependent manner. No reactivity was observed for the supernatant of CHO or MBP, which were used as controls.

Industrial Applicability

The present invention facilitates the evaluation of renal functions not by a highly invasive test like renal biopsy, but by using noninvasive samples like urine and blood. The realization of a simple *in vitro* evaluation of renal functions enables a quick and inexpensive test service that can be provided to many more subjects than before. There are many renal diseases, in which it is better to delay the initiation of dialysis treatment by an early detection of the diseases. Thus, the present invention not only eases the burden on the subjects, but also enables detection and observation of more patients who would need dialysis treatment in the future by providing methods for evaluating renal functions with simple examination procedures. Furthermore, a high evaluation reliability can be expected thanks to the excellent indicator, the megsin protein found in biological specimens like urine and blood, which reflects renal function.

Particularly, the immunological detection method of the megsin protein, and the kit used for the method, which are provided by the present invention, are superior in manipulatability utilizing magnetic granule carriers. Quick and accurate measurement of the megsin protein is enabled by the present detection method. Furthermore, since the method enables the understanding of the degree

of mesangial cell proliferation, the present invention makes it easy to determine the timing of when to commence or whether or not to continue steroid therapy used for treating IgA nephropathy.

1. 2. 3. 4. 5. 6. 7. 8. 9. 10. 11. 12. 13. 14. 15. 16. 17. 18. 19. 20. 21. 22. 23. 24. 25. 26. 27. 28. 29. 30. 31. 32. 33. 34. 35. 36. 37. 38. 39. 40. 41. 42. 43. 44. 45. 46. 47. 48. 49. 50. 51. 52. 53. 54. 55. 56. 57. 58. 59. 60. 61. 62. 63. 64. 65. 66. 67. 68. 69. 70. 71. 72. 73. 74. 75. 76. 77. 78. 79. 80. 81. 82. 83. 84. 85. 86. 87. 88. 89. 90. 91. 92. 93. 94. 95. 96. 97. 98. 99. 100.

CLAIMS

1. A method for evaluating renal functions, which comprises measuring the megsin protein in a biological specimen.
- 5 2. The method for evaluating renal functions of claim 1, which comprises measuring the megsin protein in a biological specimen, and comparing it with the megsin protein amount in a normal specimen.
3. The method for evaluating renal functions of claim 1, wherein the biological specimen is urine.
- 10 4. The method for evaluating renal functions of claim 1, wherein the megsin protein in the biological specimen is measured by an antigen-antibody reaction using an anti-megsin protein antibody.
5. The method for evaluating renal functions of claim 4, wherein the anti-megsin protein antibody is a monoclonal antibody.
- 15 6. A reagent for diagnosing renal functions, which comprises the anti-megsin protein antibody.
7. The reagent for diagnosing renal functions of claim 6, wherein the anti-megsin protein antibody is a monoclonal antibody.
8. A granule for detecting megsin protein in a biological specimen, 20 wherein the granule comprises a solid granule to the surface of which an anti-megsin protein antibody is bound.
9. The granule for detecting megsin protein of claim 8, wherein the solid granule is magnetic.
10. The granule for detecting megsin protein of claim 8, wherein the 25 relative density of the solid granule is not smaller than 1.
11. The granule for detecting megsin protein of claim 8, wherein the anti-megsin protein antibody is a monoclonal antibody.
12. A method for detecting megsin protein in a biological specimen, comprising the following steps of:
- 30 i) contacting the granule for detecting megsin protein with the biological specimen, said granule comprising a solid granule to the surface of which an anti-megsin protein antibody is bound.
- ii) contacting the second anti-megsin protein antibody bound to a marker molecule with said granule for detecting megsin protein to 35 which the biological specimen was contacted. and,
- iii) detecting the marker molecule bound to the megsin protein through

the second anti-megsin protein antibody.

13. The method for detection of claim 12, wherein the first anti-megsin protein antibody and the second anti-megsin protein antibody are both monoclonal antibodies.

5 14. The method for detection of claim 13, wherein the first anti-megsin protein antibody and the second anti-megsin protein antibody are antibodies having different recognition sites.

15. The method for detection of claim 12, wherein the biological specimen is urine.

10 16. The method for detection of claim 12, wherein the biological specimen is blood.

17. A kit for detecting megsin proteins, which comprises the following elements:

15 a) magnetic solid granules to which anti-megsin protein antibodies can be bound,

b) anti-megsin protein antibodies, which are bound to said magnetic solid granules in advance, or can be bound to them indirectly, and

c) a magnet.

20 18. The kit for detecting megsin proteins of claim 17, further comprising an anti-megsin protein antibody to which a marker molecule is bound.

ABSTRACT

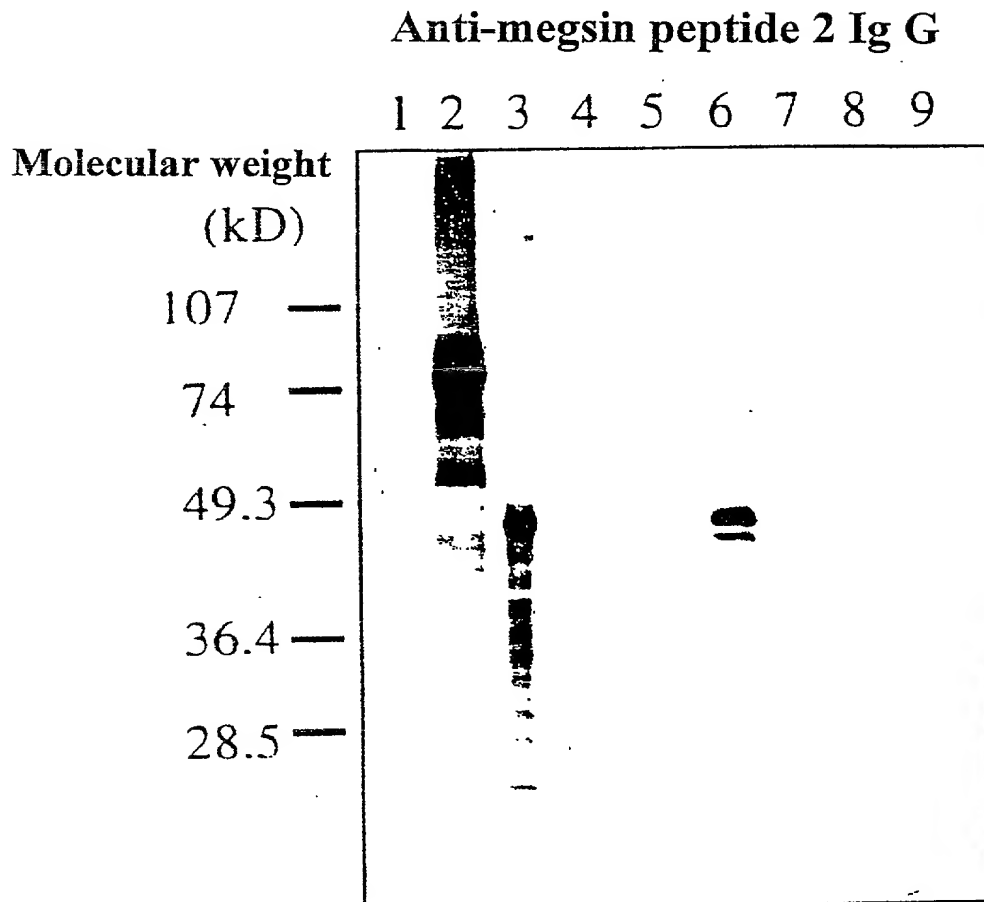
5 The present invention provides a method for evaluating renal
functions by measuring megsin protein in a biological specimen.
Moreover, the present invention provides immunological assay reagents,
as well as kits useful for the measurement of megsin protein in the
biological specimen.

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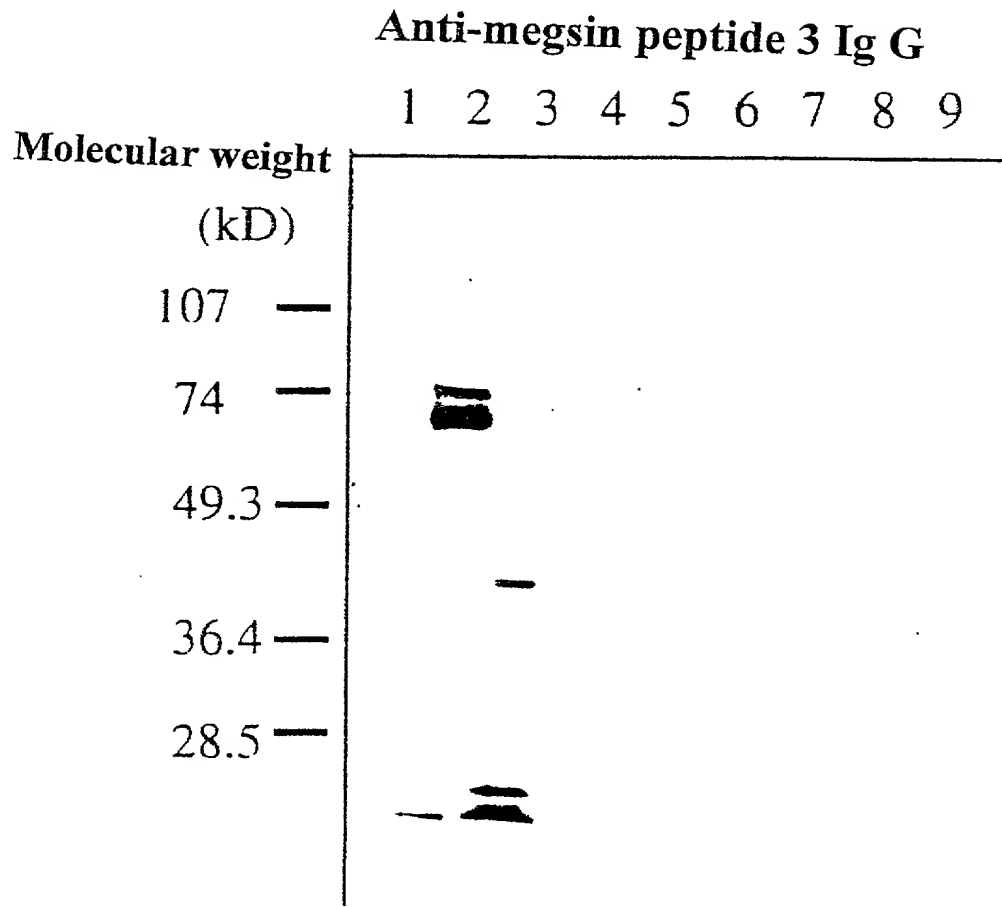
Figure 1



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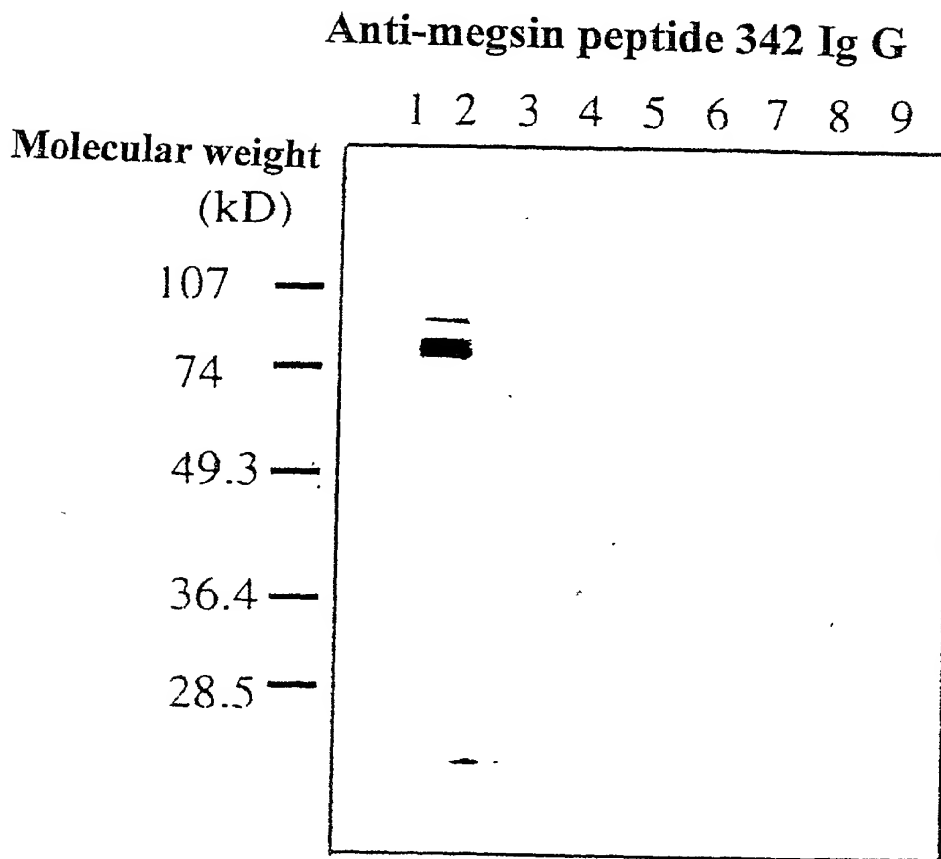
Figure 2



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Figure 3

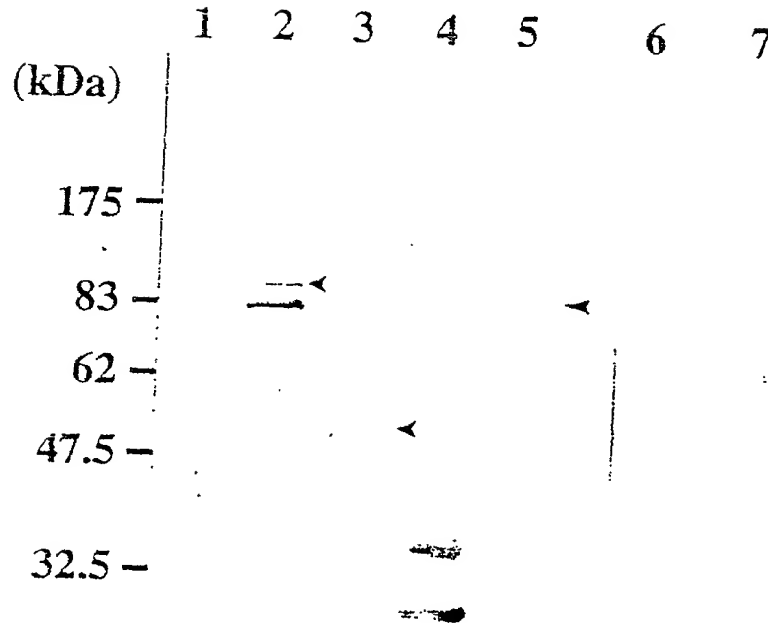


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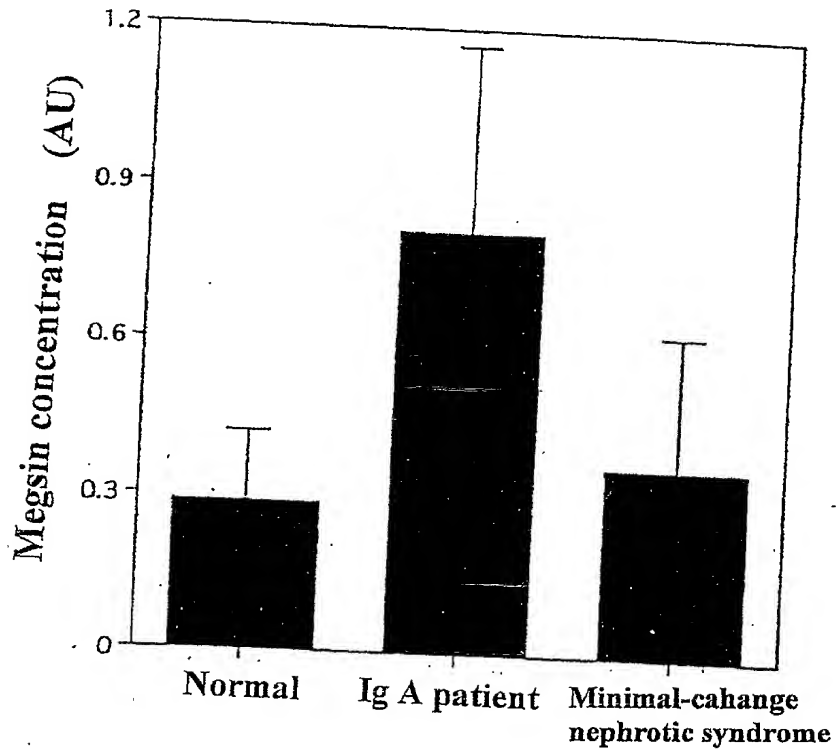
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Figure 4



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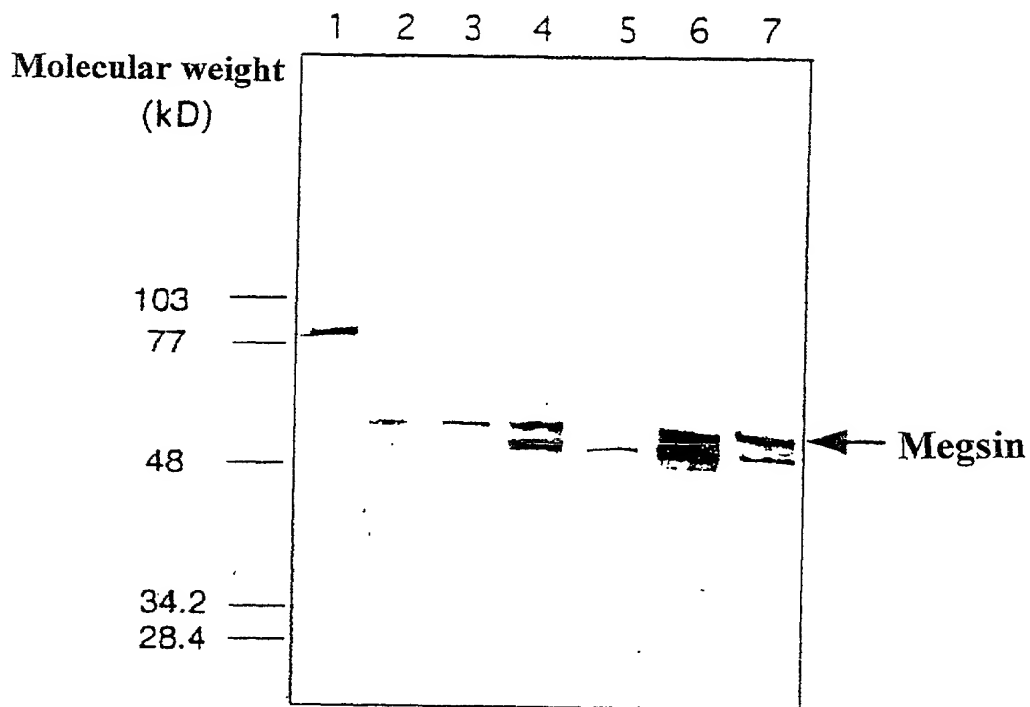
Figure 5



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Figure 6



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Figure 7

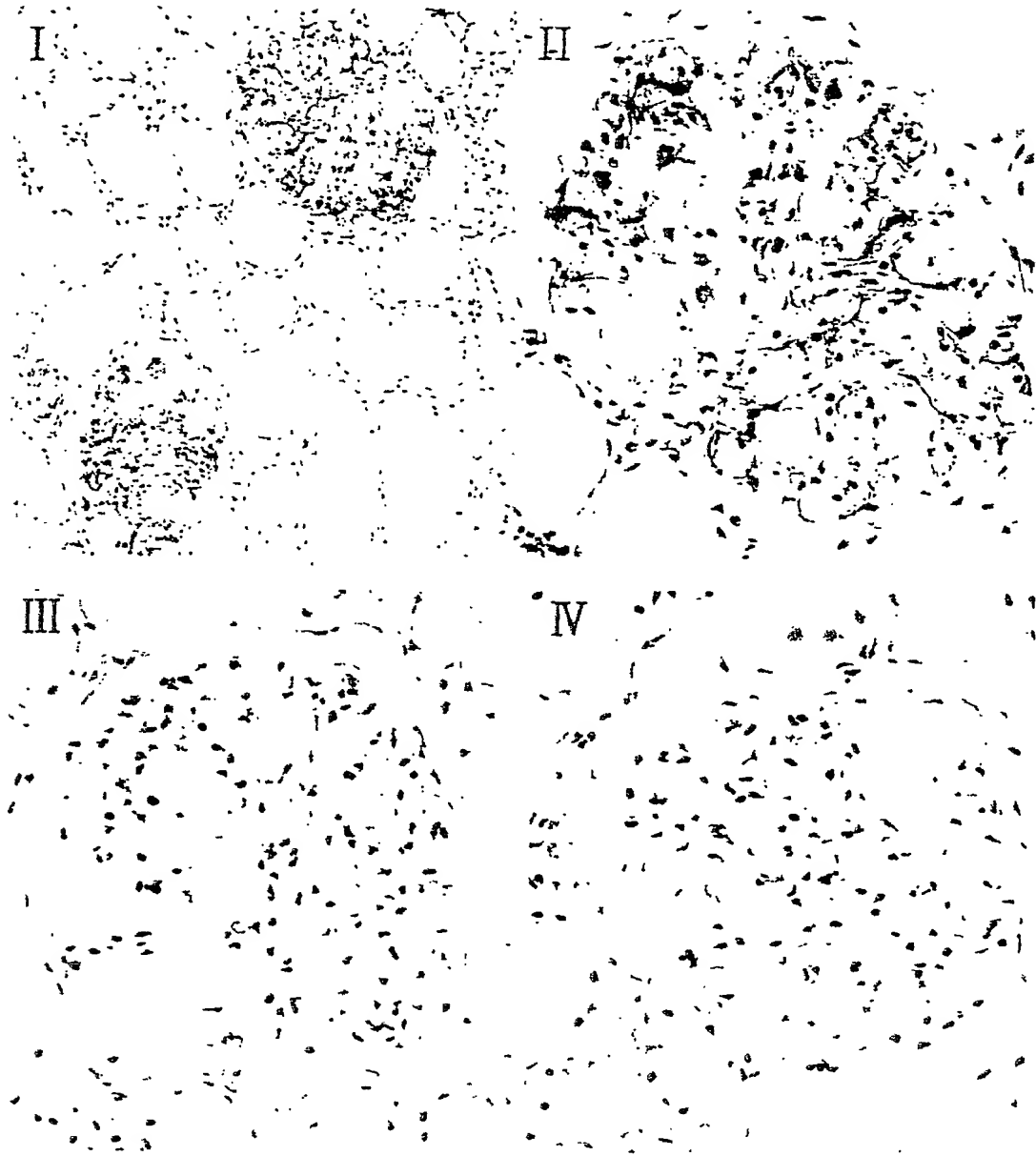


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Figure 8

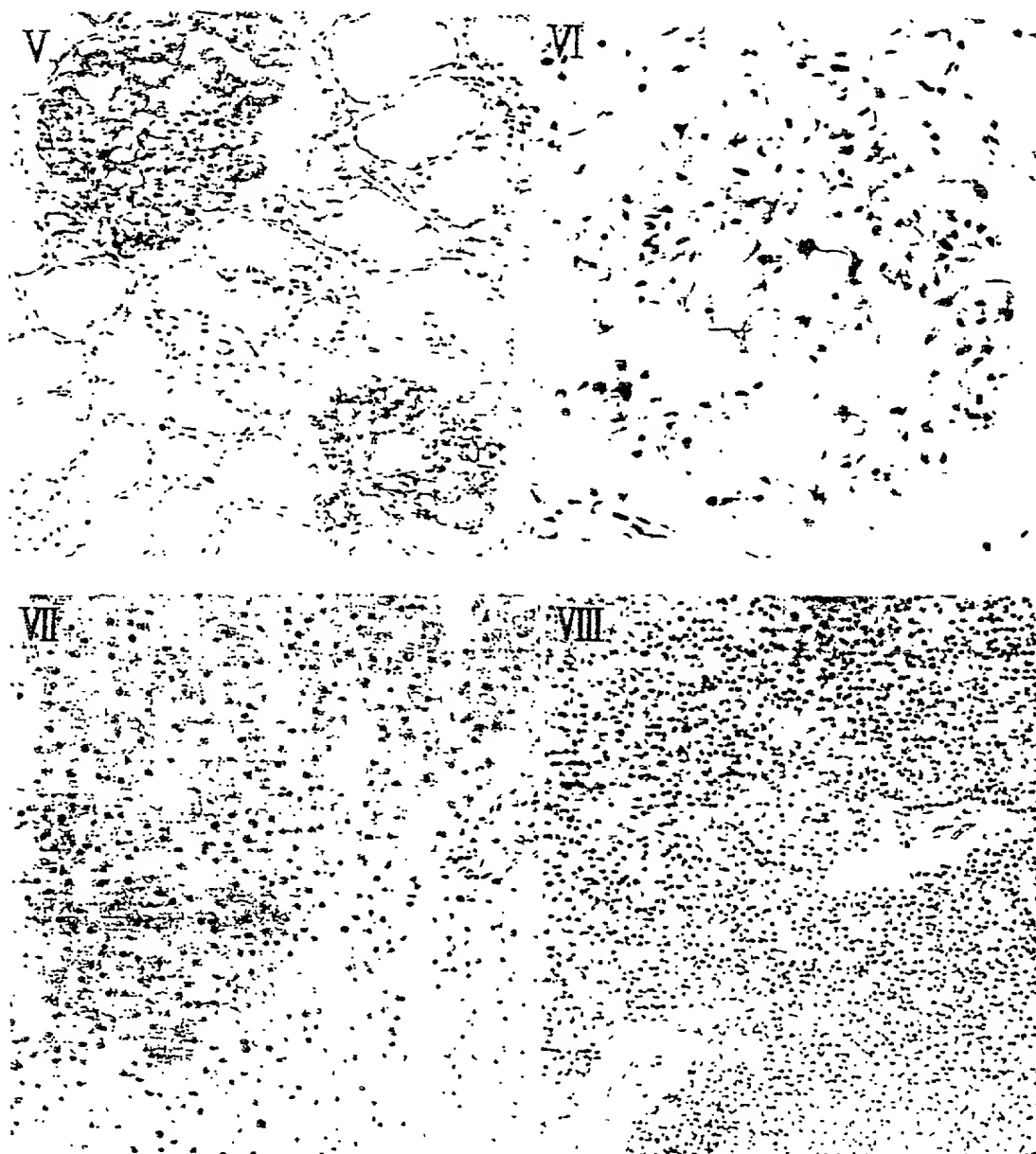


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Figure 9



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I

II

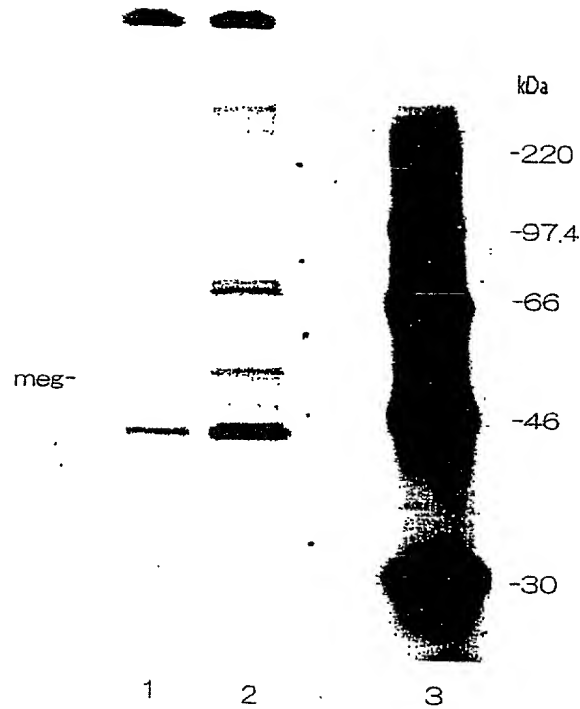
III

IV

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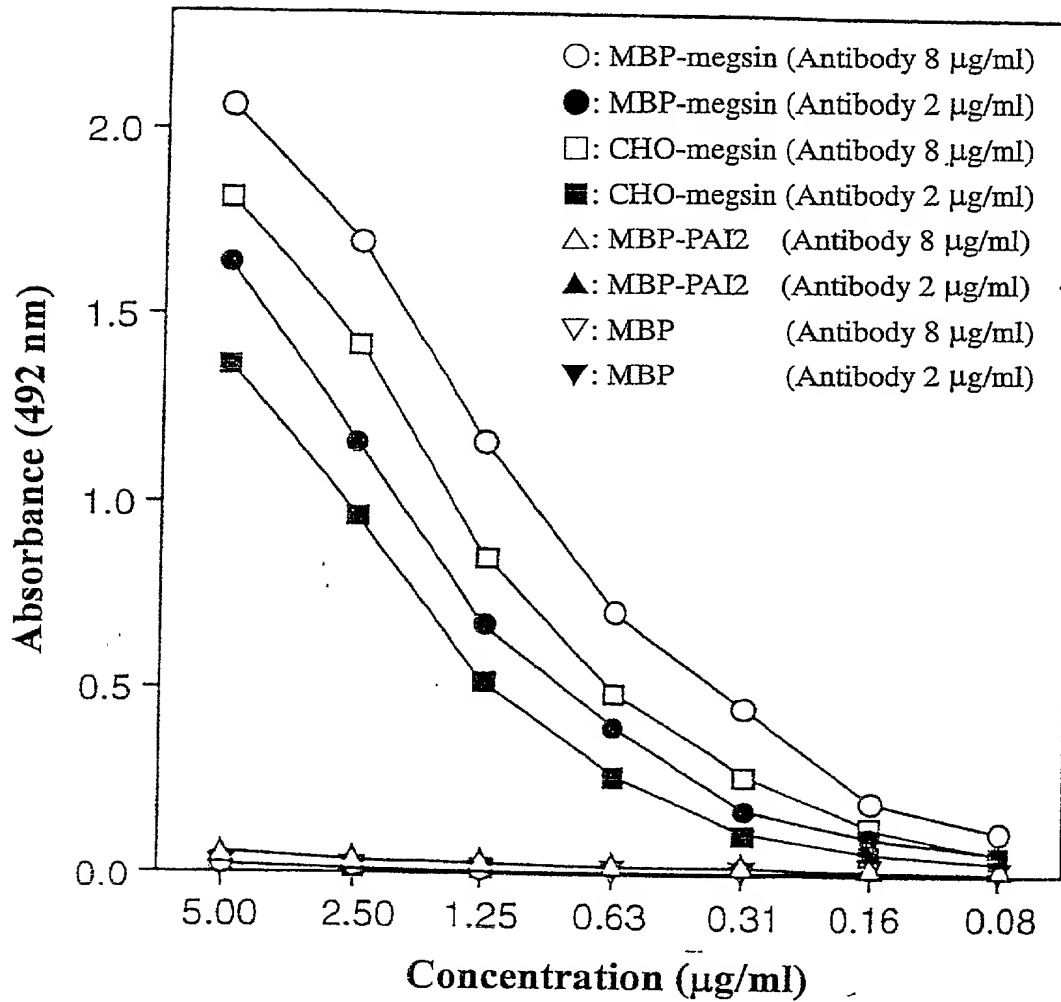
Figure 11



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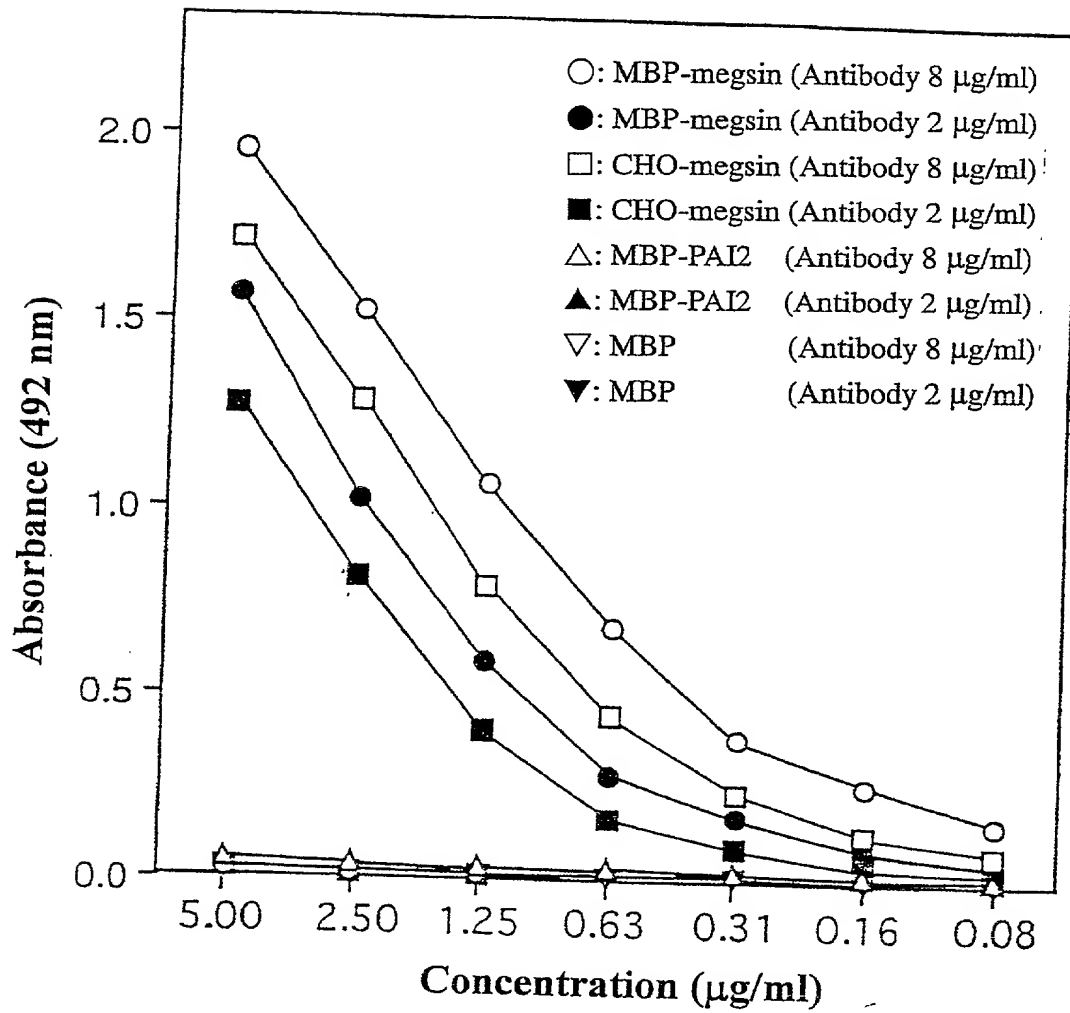
Figure 12



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Figure 13



As a below-named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

A METHOD FOR DETECTING MEGSIN PROTEIN AND USE THEREOF

the specification of which (check only one item below):

☐ is attached hereto.

☐ was filed as United States application

Serial No.

on

and was amended

on _____ (if applicable).

☒ was filed as PCT international application

Number PCT/JP00/01646

on 17 March 2000,

and was amended under PCT Article 19

on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, '1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, '119 of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed:

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|---------|-------------|-----------------|---|
| Japan | 11/75305 | 19 March 1999 | <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO |
| Japan | 11/306623 | 28 October 1999 | <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO |
| | | | <input type="checkbox"/> YES <input type="checkbox"/> NO |

Combined Declaration For Patent Application and Power of Attorney (Continued)
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POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. (List name and registration number)

Karl Bozicevic, Reg. No. 28,807
Bret E. Field, Reg. No. 37,620
Paula A. Borden, Reg. No. 42,344
Carol M. LaSalle, Reg. No. 39,740
Robert Hall, Reg. No. 39,209
David Phinney, Reg. No. P48,364

Carol L. Francis, Reg. No. 36,513
Pamela J. Sherwood, Reg. No. 36,677
Alan W. Cannon, Reg. No. 34,977
Frank Becking, Reg. No. 42,309
Kathleen Hall, Reg. No. 44,143

Send Correspondence to:

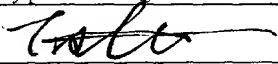
BOZICEVIC, FIELD & FRANCIS LLP
200 Middlefield Road, Suite 200
Menlo Park, CA 94025
Telephone: (650) 327-3400
Facsimile: (650) 327-3231

Direct Telephone Calls to:
(name and telephone number)

Name: Karl Bozicevic
Registration No. 28,807
Telephone: (650) 327-3400

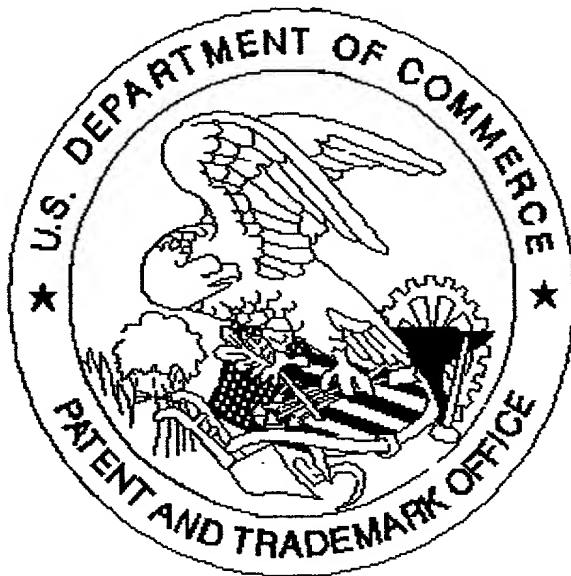
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|-----|-------------------------|--|---|---|
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MIYATA | FIRST GIVEN NAME
Toshio | SECOND GIVEN NAME |
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Kanagawa, Japan JPN | COUNTRY OF CITIZENSHIP
Japan |
| | POST OFFICE ADDRESS | POST OFFICE ADDRESS
102 Ekuseru Isehara, 16-25, Sakuradai 2-chome | CITY
Isehara-shi | STATE & ZIP CODE/COUNTRY
Kanagawa 259-1132 Japan |

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| DATE | November 19, 2001 |

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